

THE REPRODUCTIVE AND LARVAL ECOLOGY OF  
THE INTERTIDAL NUDIBRANCH MOLLUSC  
'ADALARIA PROXIMA' (ALDER & HANCOCK)  
(GASTROPODA : OPISTHOBRANCHIA)

Helen Lucy Jones

A Thesis Submitted for the Degree of PhD  
at the  
University of St Andrews



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The Reproductive and Larval Ecology of the Intertidal  
Nudibranch Mollusc  
*Adalaria proxima* (Alder & Hancock)  
(Gastropoda: Opisthobranchia)

by

Helen Lucy Jones

Thesis submitted to the University of St Andrews in candidature for the degree  
of Doctor of Philosophy.



Gatty Marine Laboratory,  
School of Biological and Medical Sciences,  
University of St Andrews.

September 1995



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This thesis is dedicated to my family.

## The Waters

The world below the brine.  
Forests at the bottom of the sea- the branches and leaves,  
Sea-lettuce, vast lichens, strange flowers and seeds- the thick tangle, the  
openings, and the pink turf,  
Different colours, pale grey and green, purple, white, and gold- the play of the  
light through the water,  
Dumb swimmers there among the rocks- coral, gluten, grass, rushes- and the  
aliment of the swimmers,  
Sluggish existences grazing there, suspended, or slowly crawling close to the  
bottom:  
The sperm-whale at the surface, blowing air and spray, or disporting with his  
flukes,  
The leaden-eyed shark, the walrus, the turtle, the hairy sea-leopard, and the  
sting-ray.  
Passions there, wars, pursuits, tribes- sight in those ocean depths- breathing  
that thick breathing air, as so many do.  
The change thence to the sight here, and to the subtle air breathed by beings like  
us, who walk this sphere:  
The change onward from ours to that of beings who walk other spheres.

Walt Whitman

## Declaration

i) I, Helen Lucy Jones, hereby certify that this thesis, which is approximately 67,000 words in length, has been written by me, that it is a record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Signed

Date 29-September '95.

ii) I was admitted as a research student under Ordinance No.12 in October, 1991 as a candidate for the degree of Ph.D. in October, 1992; the higher study for which this is a record was carried out in the University of St.Andrews between 1991 and 1995.

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iii) I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Ph.D. in the University of St.Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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## Abstract

This study concerns the reproductive and larval ecology of the nudibranch *Adalaria proxima*. Attainment of competence is demonstrated to be cue specific. Embryos metamorphose in response to choline, yet remain precompetent to elevated potassium and the natural cue until after hatching. It is hypothesized that the choline-mediated metamorphic pathway (or choline-sensitive portion of the natural pathway) becomes functionally complete ontogenetically earlier than do larval chemoreceptors.

Larvae metamorphose in response to sea water 'conditioned' (CSW) by the bryozoan *Electra pilosa*. A range of littoral organisms failed to induce metamorphosis and it is suggested that *A. proxima* displays a high degree of cue specificity. It is reported that CSW-mediated metamorphosis is dose dependent and effected in a disparate manner to that of potassium. No evidence for a bacterial rôle in metamorphosis was found. These results are intended to facilitate future isolation of the natural cue.

Both starved (lecithotrophic) and fed (facultatively planktotrophic) larvae may successfully delay metamorphosis for 28-31d post-hatching (at 10°C). Larval fitness appeared to be unaffected by nutritional status, which is suggested to reflect the transitional evolutionary nature of facultative planktotrophy. The lowered fitness commensurate with an extended pelagic period is hypothesized to confer a lowered dispersal potential.

*A. proxima* is semelparous, producing up to eleven spawn before dying. Significant variation in reproductive traits is demonstrated within and between six U.K. populations of *A. proxima*. Intrapopulation decreases in egg size, fecundity and hatching success with spawn laying sequence are suggested to reflect phylogenetic constraints. Interpopulation differences in egg size and fecundity are considered consistent with the predicted limited larval dispersal potential. Population egg size was correlated to larval size but not latitude or fecundity. Possible causative factors of the observed interpopulation variation in reproductive traits are discussed, and most probable causes hypothesized.



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## CHAPTER 1

### GENERAL INTRODUCTION

#### An Introduction to Larval Ecology

In order to understand the ecology and life history of many marine benthic invertebrates, a knowledge of the larval phase is essential. The discipline of larval ecology focuses upon this larval phase, and concerns the processes which affect patterns of larval distribution and abundance, and the study of embryonic/larval processes which subsequently affect the abundance patterns of adults (Young, 1990). The importance of larval processes was first recognized by aquaculturists, and consequently much of the early development in this branch of ecology may be traced through classical research conducted upon oyster larvae (Möbius, 1877; Montagu, 1890; Nelson, 1924, Mazzarelli, 1922; Cole & Knight-Jones, 1939). For a comprehensive overview of the development of larval ecology the reader is referred to the eclectic review provided by Young (1990). The life history strategies of marine invertebrates have subsequently been extensively investigated, particularly those of benthic molluscs (Thorson, 1946, 1950, 1957; Thompson, 1958, 1967; Vance, 1973a & b; Underwood, 1974; Crisp, 1976b, 1984).

Observing the development of invertebrate larvae from the Øresund sound in Denmark, Thorson (1946) proposed a defining categorization of larval groupings according to developmental type. Amongst the higher gastropods those species with no free veliger stage were termed direct developers, those with a short non-feeding planktonic stage lecithotrophs, and those with an extended obligatory feeding planktonic stage planktotrophs. Thorson (1950) reported the pelagic larval modes to predominate, and estimated that 70% of benthic temperate and tropical marine invertebrates produced larvae which spent some period of time in the water column. Since it is estimated that only a minority (less than half) of the larvae of benthic invertebrates have been described (Vecchione, 1985), this is difficult to verify. The original scheme proposed by Thorson (1946) was modified by Thompson (1967) who termed planktotrophs, lecithotrophs, and direct developers Types 1, 2, and 3

respectively. Egg size is a central factor in the study of life history strategies, being closely linked to traits such as fecundity and larval size (Thorson 1950; Vance, 1973a & b). Thompson (1967) noted the cline in egg sizes across the developmental types, and by connecting egg size to mode of development, he characterized Type 3 species to have fewer and more yolky eggs with a longer development period (completed within the egg) than do Type 1, with Type 2 organisms showing intermediate characteristics. Direct development among marine invertebrates is considered to be a later and more advanced evolutionary derivative of Type 1 development (Strathmann 1978, 1985; Scheltema, 1986; Kempf & Todd, 1989). Amongst opisthobranchs, evidence for an ancestral planktonic stage is displayed by the opisthobranch mollusc *Cadlina laevis*, the direct developing (Type 3) embryos of which display a vestigial embryonic stage with a velum which is resorbed before hatching (Kempf & Todd, 1989). Neither is this atypical; similar phenomena in other Type 3 developers have been also described (Tardy, 1962; Schönenburger, 1969).

#### An Introduction to Life History Theory

Life history theory is concerned with the reproductive tactics exhibited by organisms in response to sometimes conflicting evolutionary pressures. A tactic is defined as a set of co-adapted traits designed, by natural selection, to solve an ecological problem (Stearns, 1976; 1989). The key traits composing a life history tactic, as outlined by Stearns (1976), include the brood size, the size of eggs, the age distribution of reproductive effort, the effect of parental reproductive effort on adult mortality and the variation of these parameters exhibited among an individuals progeny. These traits do not exist in isolation and resources are finite - in order for one to increase there must be a trade-off at the expense of another (Lack, 1954; Cody, 1966; Charnov & Schaffer, 1973; Brockleman, 1975; Williams, 1975). Trade-offs may occur between any combination of traits limited by a finite resource such as energetic reserves or time (Lessels, 1991). There are, however, two fundamental trade-offs with which much of life history theory is concerned - the proportion of energy invested in reproduction (the 'total reproductive effort') and the allocation of this effort between progeny. The reader is referred to the work of Lack (1954) and succeeding models concerning the optimum allocation of reproductive resources (Vance, 1973a & b; Smith & Fretwell, 1974; Underwood, 1974; Brockleman,



1975; Wilbur, 1977, Parker & Begon, 1986), all of which will be discussed in Chapter Two.

### The Natural History of *Adalaria proxima*

Aspects of the general ecology and biology of the Subclass Opisthobranchia have previously been comprehensively addressed in several reviews (Bonar, 1978; Hadfield & Switzer-Dunlap, 1984; Hadfield & Miller, 1987; Havenhand, 1991). In particular, the Order Nudibranchia appears well represented by review articles within the literature (see Thompson, 1959; Harris, 1973; Bonar & Hadfield, 1974; Clark, 1975; Todd, 1981, 1983; Thompson & Jarman, 1986; Todd, 1986). The study of molluscs is facilitated by the generally well-documented, short and simple life cycles they exhibit, their accessibility for collection and the non-feeding (lecithotrophic) larval development exhibited by many species which facilitates successful laboratory culture. Nudibranch molluscs frequently display a relatively stenophagous adult diet (see review of Hadfield & Miller, 1987), and thus tend to be amenable to laboratory culture, given the proviso that the prerequisite prey species is readily obtainable. In addition, nudibranchs characteristically enclose embryos in egg capsules surrounded by a gelatinous egg mass stroma (Strathmann & Chaffee, 1984; Pechenik, 1986). The conveniently transparent nature of these masses provides the opportunity for detailed observation of egg size and embryonic development (Thompson, 1958; Harris, 1973) in addition to the quantification of fecundity.

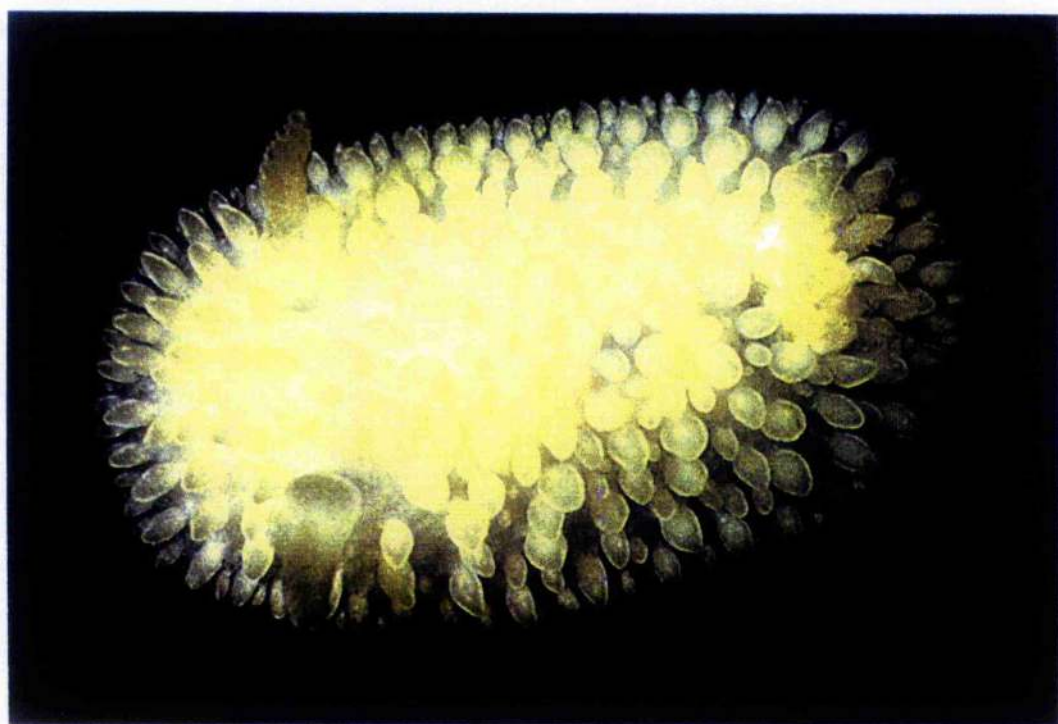
*Adalaria proxima* was first described by Alder & Hancock (1844-45) (as *Doris proxima*) in the first systematic classification of British nudibranch molluscs. The life history and developmental biology of this dorid nudibranch was first addressed in the extensive treatise of Thompson (1958a). It was this early and catholic examination of all aspects of the life history which provided the prerequisite basis of knowledge upon which later researchers have built. Subsequent research upon *A. proxima* has concerned aspects of its reproductive ecology and physiology (Thompson, 1958b; Todd, 1979; Havenhand, 1986; Todd, 1987; Havenhand & Todd, 1988b, 1989), general physiological ecology (Havenhand, 1986; Havenhand & Todd, 1988a; Todd & Havenhand, 1988), larval response to metamorphic stimuli (Todd *et al.*, 1991, Lambert & Todd, 1994) and the genetic variation of U.K. populations (Havenhand, 1986; Havenhand *et al.*, 1986; Todd *et al.*, 1988). It is not the aim of this introduction

to exhaustively review the wealth of available information concerning this species - all relevant knowledge will be addressed in detail within the relevant succeeding chapters - but rather, to present a overview of the distribution and life history.

*Adalaria proxima* is a dorid nudibranch (see Plate 1.1) typically found from the littoral zone down to a depth of 60m (Thompson & Brown, 1976). Although not ubiquitous, specimens may be sporadically abundant and the species displays a Boreo-Arctic distribution, being found in the Baltic, Faeroes, Iceland, E. Greenland and the Gulf of Maine (Thompson, 1958b) and around the coasts of Britain and Eire (Thompson & Brown, 1976), as far south as Plymouth (Garstang, 1894) and Cornwall (Hayward *et al.*, 1990). In common with other members of the family Onchidorididae, *A. proxima* adults possess lamellated, sheathless rhinophores on the anterior of the dorsum and contractile gills surrounding the anal papilla on the posterior dorsal surface (Hayward *et al.*, 1990). Mantle colour may vary from white to deep yellow or orange according to location, with the lamellae of the rhinophores and gills often being noticeably deeper in colour (Thompson & Brown, 1976; Hayward *et al.*, 1990). Identification of *Adalaria proxima* is complicated by the morphological and distributional similarity with another onchidorid nudibranch, *Onchidoris muricata* (Müller). Although *A. proxima* tends to attain a larger adult size than does *O. muricata* (17-25mm in comparison to 14mm) external anatomical differences, such as the relative position of the digestive gland, are relative and subtle (Thompson & Brown, 1976; Hayward *et al.*, 1990). Nonetheless, microscopic examination of mantle tubercules and rhinophores is frequently required in order to correctly specify individuals. Like most other onchidorid nudibranchs *A. proxima* feeds upon encrusting bryozoans, and field observations indicate the preferred diet to be the cheilostome bryozoan *Electra pilosa* which is commonly epiphytic upon the macroalga *Fucus serratus* (Thompson, 1958a & b; Todd, 1981; Todd & Havenhand, 1985; Todd *et al.*, 1991)

*Adalaria proxima* is a simultaneous hermaphrodite - during copulation internal and reciprocal fertilization occurs (Thompson, 1958a & b, Thompson & Brown, 1976). The resultant coiled gelatinous spawn masses are frequently found attached to *Fucus serratus* (L.) or upon the underside of boulders within the lower littoral zone. Spawn are produced in the spring, principally between early March and late April. Over the short reproductive period adults may lay an

**Plate 1.1.** *Adalaria proxima* (Gastropoda : Opisthobranchia). Scale bar represents 100mm. (Photograph from Picton & Morrow, 1994).



average of five or six masses, the first typically being the largest (31.7% of the total spawn production by dry weight) (Havenhand & Todd, 1988b). Food intake is apparently insufficient to meet the energetic demands of spawning; indeed, Havenhand & Todd (1989b) calculated 19% of the total reproductive output to be provided by adult degrowth (the catabolism of structural proteins). Adult weight may fall to just two thirds of the pre-spawning maximum, and such marked degrowth leads to rapid senescence and post-spawning death (Havenhand & Todd, 1989b). *A. proxima* is semelparous and strictly annual in life cycle - the adult generation exhibit total post-reproductive death, and thus no overlap of generations occurs (Thompson, 1958a & b; Todd, 1987; Havenhand, 1986). Newly spawned eggs are between 0.163mm and 0.193 mm in diameter (Thompson, 1958; Todd, 1987; Havenhand & Todd 1989) and give rise to pelagic lecithotrophic veliger larvae (Thompson, 1958a; Kempf & Todd, 1989). After a minimal precompetent period of between 24-48h larvae may settle and metamorphose in response to the primary adult prey species *Electra pilosa* (Thompson, 1958a & b; Todd & Havenhand, 1985; Todd *et al.*, 1991; Lambert & Todd, 1994).

#### Aims of This Study

This section presents the central rationale upon which this study of the reproductive and larval ecology of *Adalaria proxima* was based. The primary aims of this research were two-fold, the first being a study of the interpopulation differences in the reproductive ecology of *Adalaria proxima*. Second, attention was focused on the larval stage of *A. proxima* in order to assess factors having the potential to affect settlement and the duration of the pelagic phase, and thus the dispersal potential and recruitment patterns of the veliger larvae.

1. Whilst aspects of the reproductive biology of *Adalaria proxima* have been previously detailed, there exists no comparative data concerning the reproductive traits employed by this species between and within disparate populations. The presence of any such variation in allocation of reproductive resources was considered pertinent on two levels. First, whilst there exists evidence within the literature that intraspecific variation in egg size may affect subsequent developmental parameters (Kaplan, 1980a & b; Kraeuter *et al.*, 1982; Mashiko, 1985; Baur, 1990; Rossiter, 1991; Braby, 1994), there are no such reports of the



physiological effects of egg size upon later life history for any member of the Nudibranchia.

Second, the pattern of differential inter- and intrapopulation reproductive output was considered to be of significance and possibly indicative of the pressures acting upon the selection of reproductive traits, and thus the evolution of life history strategy (and by inference larval type) in *Adalaria proxima*. Stearns (1976, 1978, 1980) proposed the selection processes implicated in the evolution of gross life history traits ('basics' such as the possession of wings in birds, or legs in man) to be most clearly demonstrated by comparison of life history traits displayed between different taxa. However, the general differences shown by organisms in morphology, physiology and habitat become increasingly great between progressively higher taxonomic groupings. Moreover, trends in life history traits may be influenced by phylogenetic constraints between taxa rather than selection regimes, rendering the interpretation and the assessment of the significance of more subtle variations in traits (such as the trade-off between egg size and number) composing life history strategies to be inconclusive at this level (Stearns, 1976; Grant, 1983). Therefore, the study of life history parameters at the intraspecific level has been extensively employed in order to elucidate the nature of causative factors acting upon the selection of reproductive traits (Kaplan, 1980a & b; Brown, 1983; Grant, 1983; Karlsson & Wiklund, 1984; Baur, 1990; George *et al.*, 1990; Clarke *et al.*, 1991; Clarke & Gore, 1992; Qian & Chia, 1992; Baur, 1994; George, 1994; Dunn & McCabe, 1995). The value of intraspecific investigation has been proposed by Brown (1983) to convey a clearer indication of the significance of both evolutionary factors, such as genetic divergence between populations, and more proximate factors, such as environmentally induced phenotypic variation, which influence reproductive traits than can be obtained through interspecific comparison.

*A. proxima* was considered an especially suitable animal to employ for such interpopulation studies, being the only nudibranch for which genetic population data are available (Havenhand, 1986; Todd *et al.*, 1988; Todd *et al.*, 1994).

2. The pelagic period displayed by the larvae of sessile or sedentary marine invertebrates constitutes a defining parameter in the population ecology of that species. For example, the dispersal potential of a species has been correlated with the duration of the larval pelagic period, and will consequently be greater in species which possess pelagic larvae than in those which brood their young (Strathmann,

1974; Jablonski, 1986; Jackson, 1986; Scheltema, 1986; but see also Hedgecock, 1986). Indeed, it has been widely proposed that sexually produced pelagic larvae constitute the primary most significant dispersal agent for sessile/sedentary marine invertebrates (Crisp, 1974, 1976b; Christiansen & Fenchel, 1979; Jablonski & Lutz, 1983). Employing this rationale previous authors have consequently attributed dispersal potential to influence such fundamental parameters as the colonization potential, and hence, geographic range and evolutionary longevity of species (Scheltema, 1971, 1977, 1978; Jablonski & Lutz, 1983).

Within marine invertebrates, metamorphosis is not an endogenously-timed event (Pawlik, 1992), and therefore in order to recruit to the benthos larvae must become physiologically competent to respond to appropriate exogenous stimuli (Coon *et al.*, 1986; Morse *et al.*, 1988; Pennington & Hadfield, 1989; Bonar *et al.*, 1990). The total pelagic or pre-juvenile period consists of an obligatory precompetent phase, during which time larvae will be unresponsive to suitable settlement-inducing and morphogenic cues, followed by a period of competence (Crisp, 1974; Burke, 1983, 1986; Pechenik, 1990; Pechenik & Gee, 1993). The attainment of competence is considered a fundamental stage within marine invertebrate larvae, denoting the completion of essential developmental changes required for the niche shift from pelagic larva to benthic juvenile to occur (Miller & Hadfield, 1986). On attainment of competence, and in the absence of an appropriate cue, many species possess the ability to successfully delay metamorphosis for a finite period (see reviews of Thorson, 1950; Crisp, 1974; Pechenik, 1985, 1990). During the delay phase there appears a fundamental dichotomy within the Gastropoda - in contrast to the larvae of prosobranchs, which continue to grow (Pechenik, 1980, 1984; Pechenik & Lima, 1984) and which retain the shell upon metamorphosis, the larvae of opisthobranch larvae cease shell growth on attainment of competence (Kempf & Willows, 1977; Switzer-Dunlap & Hadfield, 1977; Chia & Koss, 1978; Kempf, 1981).

The total duration of the pelagic phase of a larva may therefore be affected by the length of the precompetent period, the presence or absence of the metamorphic cue, and the capacity of the larva to successfully extend the delay phase in the absence of such a cue. Accordingly, within this thesis the ontogenetic attainment of competence, the source and nature of the natural metamorphic cue, and the effective capacity of *A. proxima* to successfully extend the pelagic period are considered.

## CHAPTER 2

### INTRASPECIFIC VARIATION IN EGG SIZE

#### 2.1 INTRODUCTION

The processes of growth, maintenance and reproduction all require a proportion of an organism's finite energy reserves. To produce the maximum number of surviving offspring, these competing functions must be balanced and it is these patterns in the allocation to reproductive effort that life history theory attempts to rationalize (Winkler & Wallin, 1987). Since Fisher (1930) first addressed the question, much work has been subsequently devoted to what circumstances in an organism's environment and life history control the partitioning of resources for reproduction. Reproduction is not without cost to an organism and consequently there is a trade-off between the current reproductive success and future survival and fecundity of the adult (the reader is referred to the comprehensive review of Lessells, 1991). Selection for fewer offspring, each with a proportionally higher energy investment, will occur only if the gain in parental fitness outweighs the loss incurred by producing fewer offspring (Brockleman, 1975). The future reproductive success of an organism is negatively correlated to that organism's current reproductive effort (Williams, 1966). If the energy invested in current reproduction is increased, therefore, then the energy available for growth and maintenance is likely to be compromised, resulting in an increased risk of adult mortality and a lowering of the potential for somatic growth essential for reproduction in future breeding seasons (Williams, 1966).

Considerable inter-specific differences have been observed in the age-specific distribution of reproductive effort. Organisms may devote their total reproductive effort to spawn once and subsequently die (semelparity) or may retain sufficient energetic reserves to undergo repeated breeding (iteroparity). Cole (1954) attempted to explain the observed traits of semelparity and



iteroparity by proposing that an iteroparous organism would produce a greater number of viable offspring than would a semelparous organism, assuming the absence of juvenile mortality and the survival of every iteroparous organism to the next reproductive season. However, if this were the case, then merely by the production of one additional offspring, semelparity would become advantageous. A paucity of species displaying iteroparity relative to semelparity might consequently be expected. This is not, however, the case and the disparity between the observed frequency of iteroparous species and the number predicted by this model is known as 'Cole's paradox'. Charnov & Schaffer (1973) re-evaluated Cole's result by the inclusion of the additional considerations of adult and juvenile mortality. Iteroparity was consequently proposed to be favoured in environments where juvenile survivorship was uncertain and therefore the risk of total reproductive failure high (Holgate, 1967; Murphy, 1968; Charnov & Schaffer, 1973). Conversely, high adult mortality was predicted to select for reproduction at an earlier age and the employment of just one spawning event, i.e. semelparity (Williams, 1966; Charnov & Schaffer, 1973).

The optimal reproductive mode to be employed by an organism may be influenced by selective pressures associated with its habitat (Lessells, 1991). Stearns (1976) attempted to predict the combination of reproductive traits which would evolve in organisms subjected to certain environmental pressures. There are two theories; the deterministic (or "r-K selection") approach and the bet-hedging model. Whilst originally proposed by MacArthur & Wilson (1967) with population density as the driving selective parameter, later interpretation of the deterministic model (Schaffer, 1974) considered abiotic factors and environmental predictability to be important selective pressures. In a fluctuating, harsh, environment r-selection, which is characterized by rapid population growth (i.e. early maturity, high reproductive effort and a high number of small offspring) will be favoured. Alternatively, in a 'stable' environment biotic influences will predominate, leading to selection for the converse scenario, K-selection. The bet-hedging model is based on the survival probabilities of the juvenile and predicts that constant juvenile survivorship will favour selection for long lived, larger bodied species.

## The Evolution of Clutch Size

Investigation of the evolution of clutch size has historically constituted one of the central themes in the study of life history theory. Both clutch size and egg size are fundamental life history traits potentially affecting fitness both at the individual and the population level. Clutch size theory examines the optimum trade-off between *per capita* parental investment (and therefore, by widely accepted inference, offspring fitness) and number of offspring required in order to confer maximum parental fitness. The optimal clutch size for any organism will thus be dependent upon the total reproductive investment and the division of this effort between progeny. Because reproductive resources are subject to energetic constraints, an increase in clutch size may only be achieved by a concurrent decrease in *per capita* energetic investment. This trade-off in resource allocation was addressed by Lack's (1954) hypothesis which considers maximum parental fitness through the determination of optimal clutch size, and therefore by inference, the optimal division of resources between progeny. Other equally valid models, such as that of Smith & Fretwell (1974), take the converse approach by predicting the optimal level of investment per progeny and thence, by association, the optimal clutch size. Lack (1954) stated that reproductive processes served not the species, but the interests of the individual, and he developed what is now known as Lack's Principle, which proposed that an individual would produce the optimum number of eggs to maximize its own reproductive effectiveness, the 'Lack clutch size'. Originally formulated to explain reproductive allocation in birds (Lack, 1947, 1954), it has since been successfully applied both to other vertebrates (Kaplan & Cooper, 1984; Godfray *et al.*, 1991 and references therein) and, in particular, to marine invertebrates. Whilst Lack's clutch size theory is conceptually useful in the consideration of reproductive traits in marine invertebrates, any application is restricted by its inability to encompass the future reproductive success of an individual, considering as it does only those organisms which reproduce once within their lifetime, or those organisms for which reproduction is severely restricted. For a comprehensive review of clutch size theory the reader is referred to Godfray *et al.* (1991).

Although clutch size may be anything up to the size which maximizes clutch fitness (Lack, 1954), clutch sizes often are lower than the theoretically most productive clutch size. There are several theories which attempt to explain

this phenomenon. Among iteroparous species, lifetime fitness may be increased by a smaller than optimum clutch size which will reduce the chance of adult mortality before the next breeding season (Charnov & Krebs, 1973). In an unpredictable environment where conditions may cause total clutch mortality, it may be safer for a perennial species to 'hedge bets', thus producing a smaller than maximal clutch (Holgate, 1967). Mountford (1968) studied the normal distribution of clutch sizes and cited the decreasing probability of successful recruitment with increasing clutch size as a reason for a smaller than theoretically optimum clutch.

### The Evolution of Egg Size and Larval Type

Within evolutionary theory, egg size has been traditionally accepted as a reliable functional indication of *per capita* parental investment (Bayne, 1972; Bayne *et al.*, 1975). Egg size is a critical factor influencing the survival and growth of an individual (Mashiko, 1982). Those marine species producing small eggs will therefore produce progeny with restricted endogenous energetic reserves which consequently require to feed in the plankton before settlement (Thorson, 1946, 1950).

The Principle of Allocation, a term coined by Levins & MacArthur (unpublished, cited in Cody, 1966) and advanced by Cody (1966), proposed the energy available to an organism to be limited, and that natural selection would act in such a manner as to maximize the contribution of that organism to the succeeding generation i.e. maximize parental fitness. Since the quantity of energy available for reproduction is finite, the clutch size, or number of eggs a parent may produce, may therefore only be increased by a reduction in egg size (Nishino, 1980). Consequently, succeeding models such as those proposed by Smith & Fretwell (1974), Brockleman (1975) and Wilbur (1977) concerning the optimum allocation of reproductive resources contain four widely accepted fundamental assumptions;

1. The greater the energy invested per offspring, the greater the potential fitness of each offspring.
2. As *per capita* energetic investment increases a point of diminishing returns will be reached.

3. The number of offspring produced by a parent will tend to be inversely proportional to the energetic investment in each individual progeny.
4. There is sufficient intrapopulational variability in energetic investment per offspring to allow for change. Reproductive modes may evolve only if sufficient variability exists within a relevant trait (Kaplan, 1980).

Vance (1973a) constructed a mathematical model to predict optimal larval development mode in known environments. He assumed that the reproductive allocation of an organism was finite, and that planktotrophic larvae required less yolk (and thus *per capita* parental investment) than lecithotrophic larvae. Vance (1973a) used this model to suggest that only the extremes in egg size and development mode of lecithotrophy and planktotrophy could be evolutionary stable states. Any intermediate mode was considered to be less efficient, that is, to use more calories than the minimum required to produce a fixed number of viable offspring. In a succeeding paper Vance (1973b) further examined the stability of developmental modes and indicated that under distinct environmental conditions lecithotrophy and planktotrophy could be considered evolutionary stable strategies. He concluded that the ability of larvae to survive starvation would favour planktotrophy, and of two planktotrophs in a food limited environment, the larva with the lower ability to withstand starvation would evolve towards lecithotrophy. Whilst the validity of some of Vance's assumptions have been questioned (Underwood, 1974), evidence is lacking to either support or refute his argument (Levinton, 1982). The significant conclusion arising from Vance's rationale considered only the potential efficiency of a reproductive mode to be salient, and accepted the fundamental assumption that maximum reproductive efficiency may be conferred only by extremes in egg size. Subsequent quantitative comparative studies of reproductive energetics have allowed the testing of Vance's (1973a) theory. Todd (1979) examined the reproductive energetics of the dorid nudibranchs *Onchidoris muricata*, a planktotroph, and *Adalaria proxima*, a lecithotroph. He concluded that whilst the larger species (*A. proxima*) could employ either mode of development, the smaller body mass of *O. muricata* would allow it to employ only planktotrophy, because it lacked the energetic reserves required to meet the additional calorific demand of lecithotrophy. From this Todd (1979) proposed that it was not, as Vance had suggested, the most efficient mode of

reproduction which would be selected for, but the mode which led to the greatest absolute number of surviving offspring - even if that mode was energetically 'inefficient'. This revised view concurred with that previously stated by Stearns (1976) who pointed out that natural selection will not necessarily favour the optimal tactic but merely the best of the available options.

Chia (1974) proposed larval strategy to be dependent upon the quantity of excess energy possessed by an organism after somatic maintenance and growth requirements had been met. This was refined by Todd (1987) who stated that although selection for different larval types may be influenced by the optimization of resources to reproduction, energetics could provide only a framework, not a definitive explanation, of selection for a particular larval development mode.

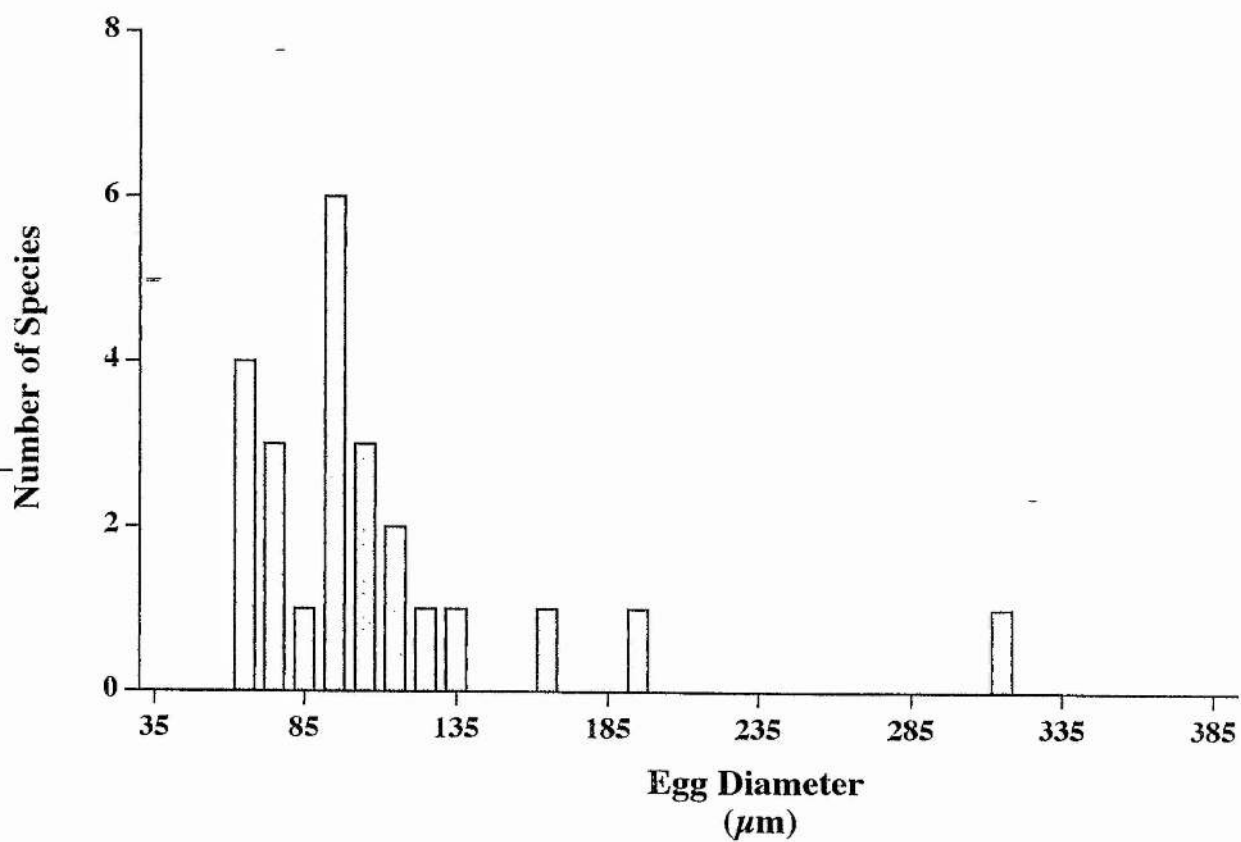
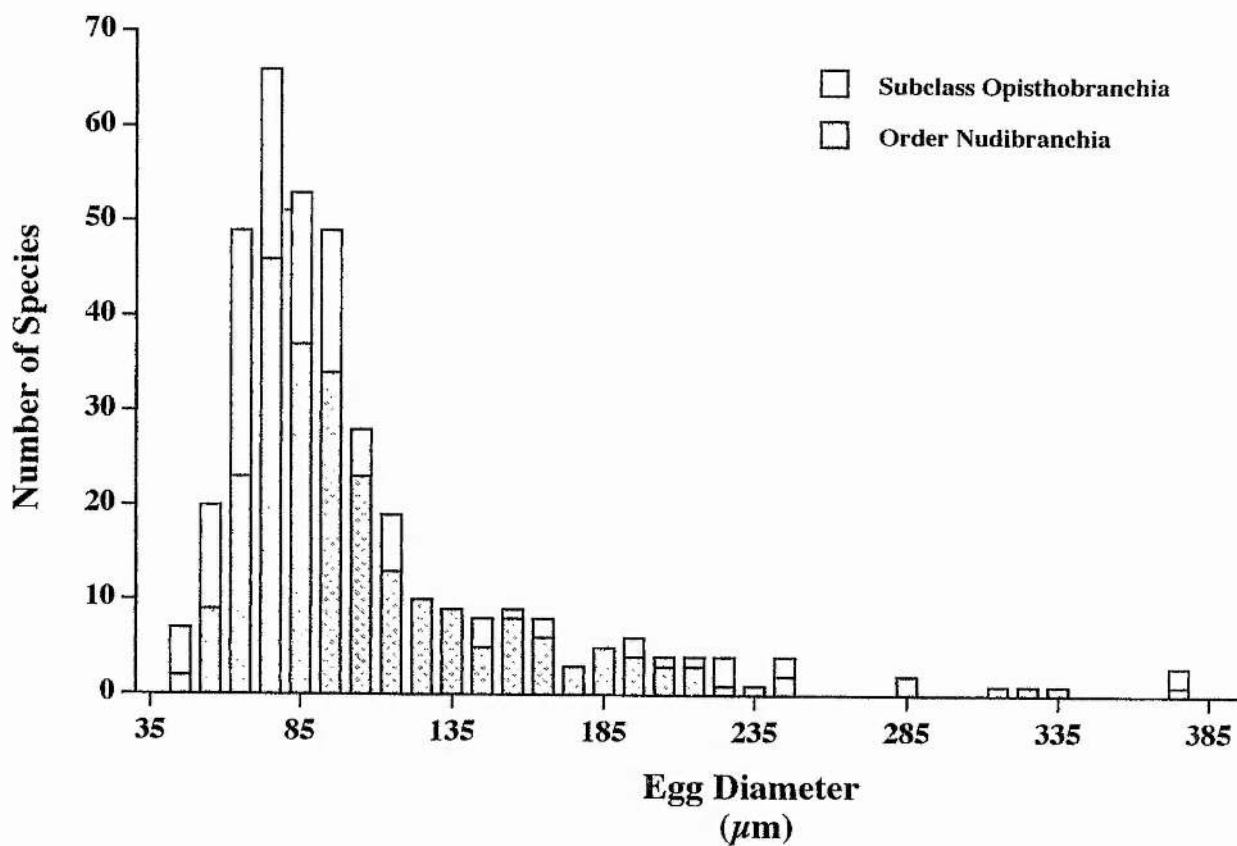
#### Egg Size and Developmental Modes Within the Opisthobranchia

The Subclass Opisthobranchia (Class Gastropoda) contains an estimated 3,000 species (Thompson & Brown, 1976) within the Orders Nudibranchia, Cephalaspidea, Sacoglossa, Anaspidea, Notaspidea and the Pteropoda. The evolution of developmental modes and egg sizes within the Opisthobranchia was considered in the comprehensive review article of Hadfield & Miller (1987)(see Figures 2.1a &b). Using pre-existing literature to obtain relevant life-history data for 369 opisthobranch species, Hadfield & Miller (1987) tested Strathmann's (1978a, 1985) theory of reproductive energetic allocation, that both the number and size of eggs, and the size of progeny at hatching will be inversely proportional to the energy invested in each egg. Small larvae may feed within the plankton in order to supplement limited endogenous reserves and equal the hatching size of offspring from species which undergo pelagic-lecithotrophic or direct development. The resultant is that all developmental modes (planktotrophic, lecithotrophic or direct developing) will result in progeny of the same size. Such 'pie' argument predictions using egg diameter to indicate relative investment per progeny failed to predict developmental mode. For example, whilst the authors did find a correlation between egg size and hatching size, no such correlation was apparent between egg size (or hatching size) and the size of newly settled juveniles, intimating the 'pie' model of reproductive resource allocation to employ some inaccurate fundamental premise(s). Neither

**Figure 2.1a. Cumulative plot showing egg size distribution within the Subclass Opisthobranchia (n=369) and the Order Nudibranchia (n=250). Adapted from Hadfield & Miller (1987).**

**Figure 2.1b. Egg size distribution within the Family Dorididae (Order Nudibranchia). Adapted from Hadfield & Miller (1987).**





was larval mode apparently predictable by consideration of reproductive effort (RE). The lack of correlation between egg size and settlement size led Hadfield & Miller (1987) to conclude there to be no single predictive factor determining developmental modes within the Opisthobranchia. Instead the authors proposed that developmental modes among the Opisthobranchia evolved in response to different selective pressures across and within opisthobranch orders. For example, selection for larger egg size may have occurred at different stages in the life history. Alternatively, during the pelagic phase high mortality in the plankton may have selected for a shorter pelagic period (and therefore larger egg size). During the benthic phase, predation on the newly settled juveniles may have selected for a larger juvenile size in order to reduce the likelihood of predation resulting, again, in a larger egg size.

#### Egg Size and Organic Content

Egg diameter has historically been regarded as a reliable functional indicator of *per capita* parental investment and, as such, has constituted a fundamental parameter in the construction of life-history models (e.g. Smith & Fretwell, 1974; Brockleman, 1975; Capinera, 1979). The putative relationship between egg size and quantity of organic material is thus a central assumption in life-history theory which has been utilized extensively both at the inter- and intraspecific level, yet may not necessarily be valid within all species. Larger eggs may, for example, merely reflect a less dense aggregation of yolk caused either during vitellogenesis or due to post-spawning hydration (Clarke, 1993). Whilst no data are apparent within the literature addressing the relationship between egg size and organic content in nudibranchs, quantitative comparative data are available for a variety of other marine invertebrate groups.

Several comprehensive interspecific studies support the validity of assuming such a link between egg size and organic content (Strathmann & Vedder, 1977; Turner & Lawrence, 1979; Kaplan, 1980b; McEdward, 1986; Goulden *et al.*, 1987; McEdward & Chia, 1991). Perhaps the most relevant to the present study is provided by Strathmann & Vedder (1977) in their comprehensive survey of the relationship between egg size (as indicated by egg volume) and organic content in members of the Echinodermata. They found a significant correlation in species with small eggs (0.05 - 0.20mm), although



larger eggs (0.70 - 0.80mm) were established to exhibit a more variable biochemical composition (Turner & Lawrence, 1979). Between species of marine invertebrates therefore (in addition to other taxa), egg size is widely accepted to be a reliable indicator of parental investment per progeny.

At the intraspecific level, where quantitative differences in egg size and content tend to be closer to the limits of experimental resolution, evidence for a relationship between egg size and organic content appears more equivocal. Significant correlations have been established within several taxa, for example, among members of the Echinodermata (McEdward & Carson, 1987; McEdward & Chia, 1991), and both decapod (Clarke *et al.*, 1991; Clarke, 1993) and isopod (Clarke & Gore, 1992) Crustacea. Equally, however, reports exist of species in which no such significant relationship between egg size and organic content is apparent; examples include members of the Echinodermata (McEdward & Coulter, 1987; and see reservations of McEdward & Carson, 1987). The putative relationship between egg size and organic content within species is discussed in detail within Section 2.4.

#### Intraspecific Variation in Egg Size

A gradual increase in egg size, concurrent with a decrease in clutch size, is regarded as a temporally progressive evolutionary trend (Mashiko, 1990), and it has been proposed that natural variation in intraspecific egg quality may have been a contributing factor in this evolutionary shift from planktotrophic to lecithotrophic development for many species (Kempf & Todd, 1989; Hadfield & Miller, 1987). These differing reproductive strategies may evolve only if, in a population, there is sufficient investment in offspring variability (Kaplan, 1980). Although selection will act upon individuals, the measurement of life history strategies can be performed only on populations because it is the variation between organisms which is the most important element of a strategy (Stearns, 1976).

Variability in egg size has been proposed to constitute an adaptive response to increase individual fitness in the face of both temporal and spatial environmental change (Thorson, 1950; Lack, 1954; Capinera, 1979; Kolding & Fenchel, 1981; Kraeuter *et al.*, 1982; Kaplan & Cooper, 1984; Baur, 1994;

Dunn & McCabe, 1995). Alternatively, such variability may be a non-adaptive phenomenon reflecting merely weak selection (McGinley *et al.*, 1987) or the physiological inability of the adult to accurately partition reproductive resources during vitellogenesis (Qian & Chia, 1992; Karlsson & Wiklund, 1984, Baur, 1994).

Gadgil & Solbrig (1972) and Smith & Fretwell (1974) analysed the trade-off between offspring size and number and concluded parental fitness to be maximized when the investment in each of the offspring is equal. However, a greater degree of egg size variation has been observed within individual spawn masses than might be predicted from such an optimality theory (Thompson, 1958, 1967; Kerfoot, 1974; Capinera, 1979; Brady & Lawlor, 1984; Karlsson & Wiklund, 1984; McEdward & Carson, 1987; Parker & Begon 1987, Seigel & Ford, 1992) For example, McEdward & Carson (1987) found egg sizes within individual spawn masses of the starfish *Solaster stimpsoni* to vary by as much as 39% from the mean, and energy content to vary by up to 48%.

Not only may egg size vary within a single mass of eggs but also, in those species which spawn more than once, between successive spawn masses within a season. Such seasonal variation in egg size between successive spawn masses laid by an individual has been documented in vertebrates, e.g. fish (Blaxter & Hemple, 1963) and amphibians (Kaplan, 1980). The phenomenon also is apparent among the invertebrates and has, for example, been reported to occur within the classes Insecta (Karlsson & Wiklund, 1984); Gastropoda (Baur, 1990, 1994), Bivalvia (Kraeuter *et al.*, 1982), Crustacea (Nishino, 1980; Mashiko, 1982, 1986, 1990; Clarke *et al.*, 1991; Dunn & McCabe, 1995); Parker & Begon, 1986) and Polychaeta (Qian & Chia, 1992).

Intraspecific variation in egg size may also occur at the interpopulation level, between geographically separated populations. Such differences in egg size over the distribution range of a species have been established to occur within the Echinodermata (George *et al.*, 1990; George, 1994) and have been extensively reported within the Crustacea - e.g. Isopoda (Clarke & Gore, 1992); Amphipoda (Dunn & McCabe, 1995); Cirripedia (Patel & Crisp, 1960); Decapoda (Nishino, 1980; Mashiko, 1982, 1990; Clarke *et al.*, 1991). The phenomenon of intraspecific variation in egg size and associated causative factors will be addressed in Section 4.4.

### The Influence of Egg Size on Later Life History

Intraspecific studies performed upon a range of invertebrate species have established that egg size has the potential to affect subsequent life history parameters. Several useful studies of egg size have been performed among the Class Insecta which show that egg size may significantly affect subsequent offspring fitness components such as time to hatching, developmental rate and hatching success (Rossiter, 1991; Braby, 1994; Fox, 1994). However, there are examples of egg size having no significant effect on the subsequent performance of progeny (e.g. Karlsson & Wikland, 1984; Braby, 1994). It has been proposed that, in some species, the adaptive significance of egg size variation may only become apparent under certain environmental conditions. For example, Braby (1994) showed, by varying the food quality to which three species of butterfly (*Mycalesis perseus*, *Mycalesis terminus* and *Mycalesis sirius*) were subjected, that the adaptive advantage of larger egg size was affected by larval environment. There was a positive relationship between egg size and offspring quality within all species treated with the poor quality diet, yet the fitness advantage associated with larger eggs was retained within only one species (*M. perseus*) when subjected to the higher quality diet. The significance of egg size variation on offspring performance may therefore be apparent only in suboptimal environments (low food quality or starvation, increased competition) within some species (Fox, 1994). Thus, it is possible that by applying optimal husbandry and culture techniques, laboratory studies may mask the very effects they are attempting to study.

The duration of the embryonic period is an important and characteristic embryological parameter within each species. If ovum size does influence developmental time then in an environment where hatching time is important, variability in ovum size will have the potential to affect offspring fitness (Kaplan, 1980). Whilst a large egg may result in a large larva, yolk hinders the penetration of cleavage furrows, reducing the rate of cleavage and resulting in an extended embryonic period (Thompson & Jarman, 1986). Vance (1973a) constructed a model relating the total larval development period (T) to the average energetic content of an egg (s) for a species by the equation ;

$$T = b - (b - a) s$$

where  $a$  and  $b$  were constants influenced by environmental variables such as water temperature and salinity. Whilst subsequent comparison of interspecific data by Underwood (1974) did not offer support for this putative relationship, the findings of later interspecific studies by Steele (1977), Thompson & Jarman (1986) and Hadfield & Miller (1987) have been consistent with the predictions of Vance (1973a). An extended embryonic period may not be desirable, conferring as it does a greater risk of mortality by both mechanical damage and by predation (Thompson & Jarman, 1986). The duration of the embryonic phase for a species will therefore be a trade-off between the optimum energetic investment per egg and the consequent length of the development period (Thompson & Jarman, 1986). Yolk content may be reduced, and thus embryonic period shortened, by the production of a smaller egg, or by several strategies in which extra yolk is supplied from the surroundings of the embryo, ensuring that cleavage rate is not slowed and yet that offspring are supplied with extra energetic reserves. Such tactics shown by the Mollusca include the predation of sibling embryos and provision of extra-zygotic albumen or extracapsular yolk (Thompson & Jarman, 1986).

Although Kaplan (1980a) found no relationship between ovum size and subsequent development in the salamander, he did find a positive correlation between ovum size and size at hatching and suggested that potential advantages associated with increased larval size act after hatching, when the young start feeding. Larger larvae may hatch at a competitive advantage, leading to an increasing disparity in sibling larval sizes - the so-called 'hierarchy' effect (Begon, 1985). Thus even a minimal difference in size at hatching may be progressively amplified by size specific growth rate and conditions at the time of growth (e.g. competition, predation or population density effects) might consequently significantly affect offspring fitness (Kaplan 1980a). A comprehensive survey of egg sizes and hatching sizes within the Opisthobranchia led Hadfield & Miller (1987) to propose species egg diameter as a reliable indicator of larval length at hatching. However, within the same data set, no such relationship was apparent at the interspecific level between egg size and settling size.

An increased egg size will be of additional ecological significance if a larger size at metamorphosis is conferred upon progeny originating from larger eggs. It has been proposed that an increased size at metamorphosis may confer

competitive advantages to an individual (Spight, 1976; Rivest, 1983). Indeed, several authors have predicted a decrease in the probability of mortality by micro-carnivore predation with increasing metamorph size (Spight, 1976; Rivest, 1983; Werner, 1986; Hadfield & Miller, 1987; Rowe & Ludwig, 1991).

### Rationâle

The rationâle underlying this section of the study was to determine whether intraspecific variation in reproductive output (egg size and number) was evident within and between populations of the dorid nudibranch *Adalaria proxima* and whether any such variation had a significant effect on later life history parameters. Interpopulation differences in reproductive parameters were therefore investigated at eight intertidal sites throughout the distribution range of this species around the northern coast of Britain (N.E. England, Scotland, Wales and N. Ireland). The primary objectives addressed included :

1. There have been no previous reports of whether *Adalaria proxima* exhibits differences in egg size or number over its distribution range. The initial objective therefore was to determine whether any such interpopulation differences were apparent.
2. Life-history models assume the presence of sufficient variation in reproductive parameters within a population to allow for selection to act and thus to drive the evolution of reproductive strategies, such as the shift from planktotrophy to lecithotrophy. The pattern of resource allocation to reproductive output over the course of the reproductive season was therefore examined within each population. Possible causes of any such observed variation were investigated in order to elucidate possible determining factors affecting the division of reproductive effort between egg size and number within *Adalaria proxima*.
3. There exists evidence within the literature that intraspecific variation in egg size may affect subsequent developmental parameters, although there are no previous reports concerning the physiological effects of egg size on later life history within the Nudibranchia. The effect of egg size variation on embryonic period, hatching success and larval shell size at both the inter- and intrapopulation levels was therefore empirically determined.



## 2.2 METHODS

### Collection and Maintenance of Adults and Spawn Masses

Spawn masses were available from juveniles collected from six intertidal populations around the British Isles, and which comprised a component of a separate project (C. D. Todd & W. J. Lambert). Those juveniles were obtained in July 1992, a few weeks after settlement, and reared to sexual maturity under ambient conditions (E. Scotland; temperature and photoperiod) within through-flow trays. The 20 individuals per population were maintained in five tanks in a randomized block design, with each Teaboy (containing an isolated individual *Adalaria proxima*) retained in position with a dulled perspex rack. Populations originated from Portaferry, Northern Ireland; Menai Bridge, Anglesey, Wales; Robin Hood's Bay, North Yorkshire, England; Loch Eriboll, Sutherland, Scotland; Cuan Ferry, Argyll, Scotland, and Kinkell Braes, Fife, Scotland (the geographic locations of study sites are illustrated in Plate 2.1).

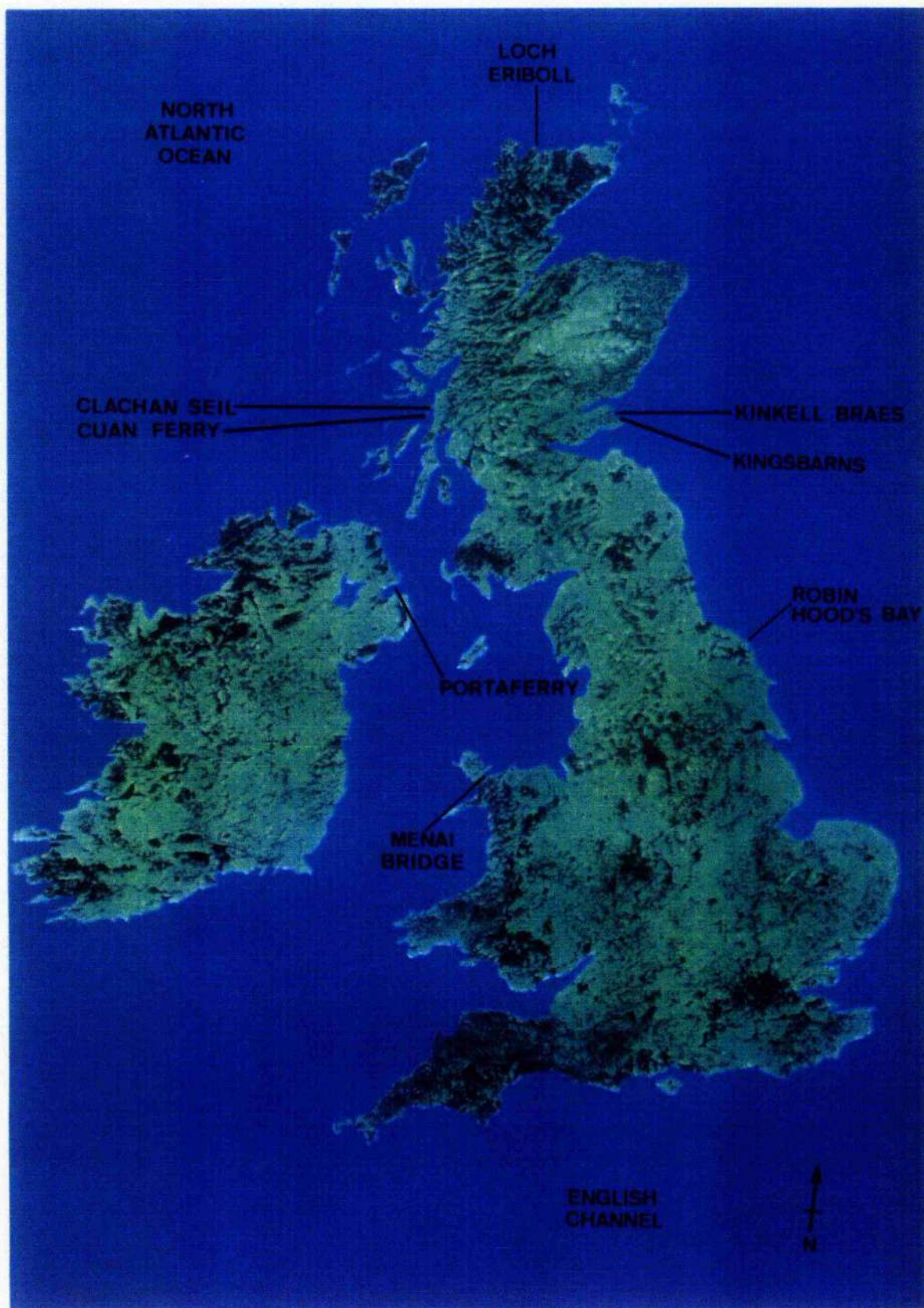
Additional pre-spawning adults were obtained from Clachan Seil, Argyll (see Plate 2.2a & b), and Kingsbarns, Fife (see Plate 2.3a) in January 1993 and were subjected to the same culture conditions as the above populations. Because individuals were not reared within the laboratory these two populations are included for their first spawn mass egg sizes only and are not incorporated into any subsequent analysis.

Adults from all eight populations were isolated before the spawning season commenced using 'Toby Teaboys®' (Aldridge Plastics Ltd.) and were supplied with fresh food *ad libitum* (*Electra pilosa* epiphytic upon *Fucus serratus*). The Teaboy design is such that a flow through of water is ensured through the clean mesh walls of the container. Adults were paired (within populations) several times a week to allow frequent copulation throughout the spawning season. Pairing combinations were such that each individual could be identified by size, body colour or, on examination under a binocular microscope, by the number and position of tubercles on the anterior dorsal surface. This allowed reliable re-isolation.

**Plate 2.1. Map of the British Isles showing the locations of sites from which *Adalaria proxima* were obtained during July 1992 - January 1994. The eight populations for which reproductive data were obtained were:**

Menai Bridge, Anglesey, Wales	53°14'N 4°9'W
Portaferry, County Down, Northern Ireland	54°24'N 5°26'W
Cuan Ferry, Argyll, Scotland	56°12'N 5°30'W
Clachan Seil, Argyll, Scotland	56°16'N 5°37'W
Loch Eriboll, Sutherland, Scotland	58°30'N 4°42'W
Kinkell Braes, Fife, Scotland	56°22'N 2°47'W
Kingsbarns, Fife, Scotland	56°20'N 2°39'W
Robin Hood's Bay, North Yorkshire, England	54°30'N 0°40'W

Satellite image adapted from 'Great Britain & Ireland Satellite Mosaic', Tony Stone Library (Published by Sunset Posters, Killiney, Ireland).





**Plate 2.2a (above) & b (below). Clachan Seil, Argyll, Scotland**  
**(56°16' N, 5°37' W) at spring low tide.** This site constitutes a tidal narrow 1km long with a maximum width of 30m bounded by a tidal sill near Clachan Bridge in the south-west to a tidal sill in the north-east. It is the presence of these sills which prevents the narrows becoming emersed, even on low spring tides (Todd & Turner, 1986). The shore is very sheltered, being bordered by elevated land on both the Argyll mainland and Seil Island shores.

a). Looking north-east towards the sill which marks the end of the tidal narrow . The Argyll mainland shore is on the right side of the photograph and Seil Island shore is on the left. The large rocky boulders and rich macrofaunal vegetation (esp. fucoids and laminarians) characteristic of this site are clearly visible.

b) Looking south-west towards Clachan Bridge. The Argyll mainland is on the left side of the photograph and the Seil Island shore is on the right.

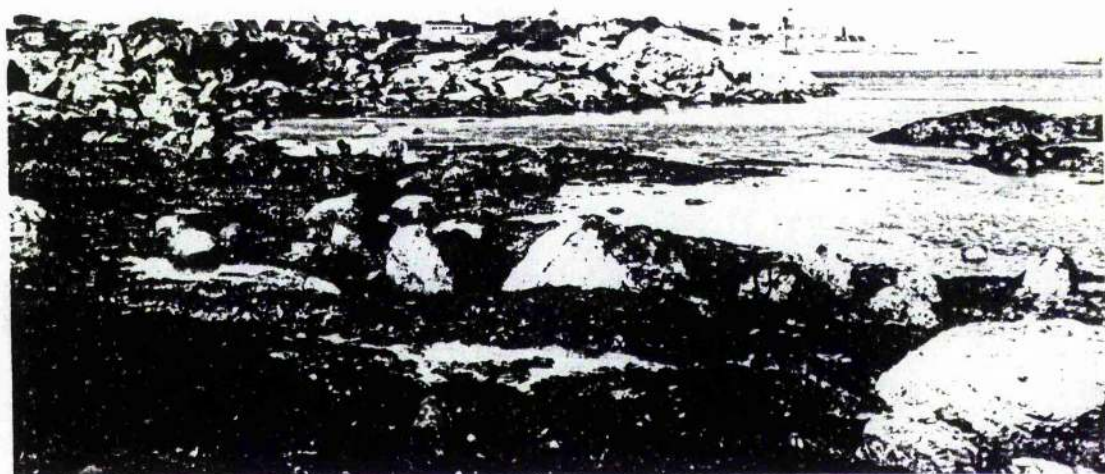


**Plate 2.3a (above).** Kinkell Braes, St. Andrews, Fife, Scotland (56° 22' N, 2° 47' W).

This rocky shore site lies within St. Andrews Bay close to the Gatty Marine Laboratory. This area is comprehensively addressed in 'Fauna and Flora of St. Andrews Bay' (Laverack & Blackler, 1974).

**Plate 2.3b (below).** Kingsbarns, Fife, Scotland (56° 20'N 2°39'W).

The site is a continuation of the rock formations evident at Kinkell Braes and consists of rocky outcrops bordered by sandy areas (Laverack & Blackler, 1974).



The resultant spawn masses, which are generally found laid upon *Fucus serratus* in the field, and on both macroalgal surfaces and the mesh container sides in the laboratory, were carefully excised and isolated each day. Upon collection each spawn mass was inspected under a Wild-E binocular microscope and, where not precluded by cell division, zygote diameters were measured using the photographic protocol detailed below. The spawn masses were then cultured in 6-well flat bottomed polystyrene tissue culture plates (diameter 3cm, depth 2cm, volume 10ml, Corning Ltd., N.Y.) and incubated (Gallenkamp cooled incubator, Sanyo Gallenkamp PLC) under constant light at 6°C in 0.45µm Millipore filtered seawater which was changed daily.

#### **Photographic Protocol for the Measurement of Zygote Diameter and Spawn Mass Egg Numbers**

This study concerned not only differences in zygote diameter between and within populations, but also the possible effects of these differences on later developmental parameters. Consequently the procedure for the determination of zygote diameter was required to be both accurate and, to minimize the possibility of temperature shock upon eggs, as rapid as possible. The direct determination of egg diameter by a compound microscope equipped with an ocular micrometer was considered suboptimal. This was primarily because of the time period required for repeated determinations of egg size to be made within each spawn mass under a relatively concentrated transmitted light source at room temperature (or above) which may have increased the probability of temperature shock. In addition, enumeration of the number of eggs contained within each spawn mass was also considered an important parameter and the time required for all eggs to be counted may have increased the probability of temperature stress. A photographic protocol was therefore devised in order to allow accurate and repeated observations of diameter to be performed with the minimum of handling. *Adalaria proxima* eggs are laid within a gelatinous spawn mass matrix, or stroma, and complete separation of the eggs from this surrounding gelatinous matter is extremely difficult to achieve without damage to the eggs (pers. obs.). Indeed, rupture of the external spawn mass membrane frequently appears to result in compromised embryonic development (C. D. Todd, pers. comm., & pers. obs.). The integrity of spawn masses was therefore maintained throughout the course of the study until naturally destroyed by the onset of larval hatching. Zygotes held within the spawn mass



were photographed under a Wild-E binocular microscope fitted with a Wild HV100 photo tube attachment and Olympus OM-2 35mm camera. Each spawn mass was placed within a glass petri dish and held under a glass cover-slip to gently flatten it. The specimen was illuminated using both transmitted and incident light (Schott KL 1500-T cold light source). Each spawn mass was initially photographed at low power in order to allow the later determination of egg numbers. High power magnification was then applied in order to determine zygote diameter. Magnification was kept constant throughout the photographing of zygotes and scale was determined by regular photographing of a slide micrometer (the same slide micrometer was used each time). Photographs were taken using Kodak Technical Pan Professional (ISO 25/15°C) black & white film and the negatives developed using AGFA Rodina black & white film developer. The resulting negative films were enlarged using a Durst M700 35mm negative enlarger. The density of egg distribution within the stroma number varied both within and between populations, therefore the number of eggs in each spawn mass was determined separately using a hand held counter. The maximum diameters of 55 zygotes per spawn mass in *ad hoc* positions were measured using digital vernier calipers (Mitutoyo Digimatic Caliper, Series 500, 150mm, repeatability rating 0.01mm). The size of the projected negative image was such that 0.01mm caliper reading was equivalent to change of 0.05µm egg diameter. The precision of the photographic protocol was assessed by the repeated 'blind' measurement of single zygotes thirty times. This was repeated using known zygotes and the maximum error incurred was found to be 0.2µm, which constituted a 0.014% error. The mean egg diameters of a total of 259 spawn masses obtained from eight U.K. populations of *Adalaria proxima* were determined within the present study.

Preliminary analysis of *Adalaria proxima* eggs examined at the zygote stage showed the egg to be spherical. Whilst it is possible that treating all eggs as spheres could introduce a degree of error, throughout the study egg shape did not appear to change and occasional abnormal eggs are readily recognized and were not measured. The equation used to convert egg diameter measurements to volume was;

$$\text{Egg volume} = \frac{4}{3} \pi r^3$$

### **Determination of Embryonic Period**

The embryonic development time and hatching success of spawn masses from each population were recorded to assess the relationship between zygote diameter, embryonic development rate and hatching success. The date on which spawn masses were laid was noted and the progress of embryogenesis regularly monitored through rapid inspection under a Wild-E binocular microscope. Hatching in *Adalaria proxima* generally occurs over a period of several days with a small number of larvae emerging on the first day and the majority rapidly emerging on the following one or two days. The recorded 'Embryonic Period' within the present study was defined as the number of days at field ambient temperature (6°C) between the spawn mass being laid to when the majority (>50%) of larvae emerged from the spawn mass.

### **Determination of Hatching Success**

'Hatching Success' within the present study was defined as the percentage of embryos within each spawn mass which successfully hatched as veliger larvae. Spawn masses were retained in culture and monitored until all healthy embryos had either eclosed or died. The typical sources of pre-hatching mortality included unfertilized eggs, early aborted development at the 16-32 cell stage and the death of advanced (veliger-stage) embryos. On inspection under a Wild-E binocular microscope the evacuated egg capsules were clearly visible, as were the remains of unsuccessful embryos. The proportion of unsuccessful progeny to successfully hatched veligers could thus be reliably determined and was expressed in percentage terms.

### **Determination of Larval Shell Length**

*Adalaria proxima* possesses a coiled, sinistral shell classified as a 'Type B' by Thorson (1946), although the later classification as a 'Type 1' shell provided by Thompson (1961) appears more widely used. Plate 2.4 illustrates a swimming veliger larva with attached shell. The photomicrograph was obtained using a Leitz Diaplan microscope with a Wild Photoautomat MP545 attachment. The veliger was anaesthetized before photography by the addition of 15% magnesium chloride in

**Plate 2.4. Photomicrograph of a Veliger Larva of *Adalaria proxima*.**

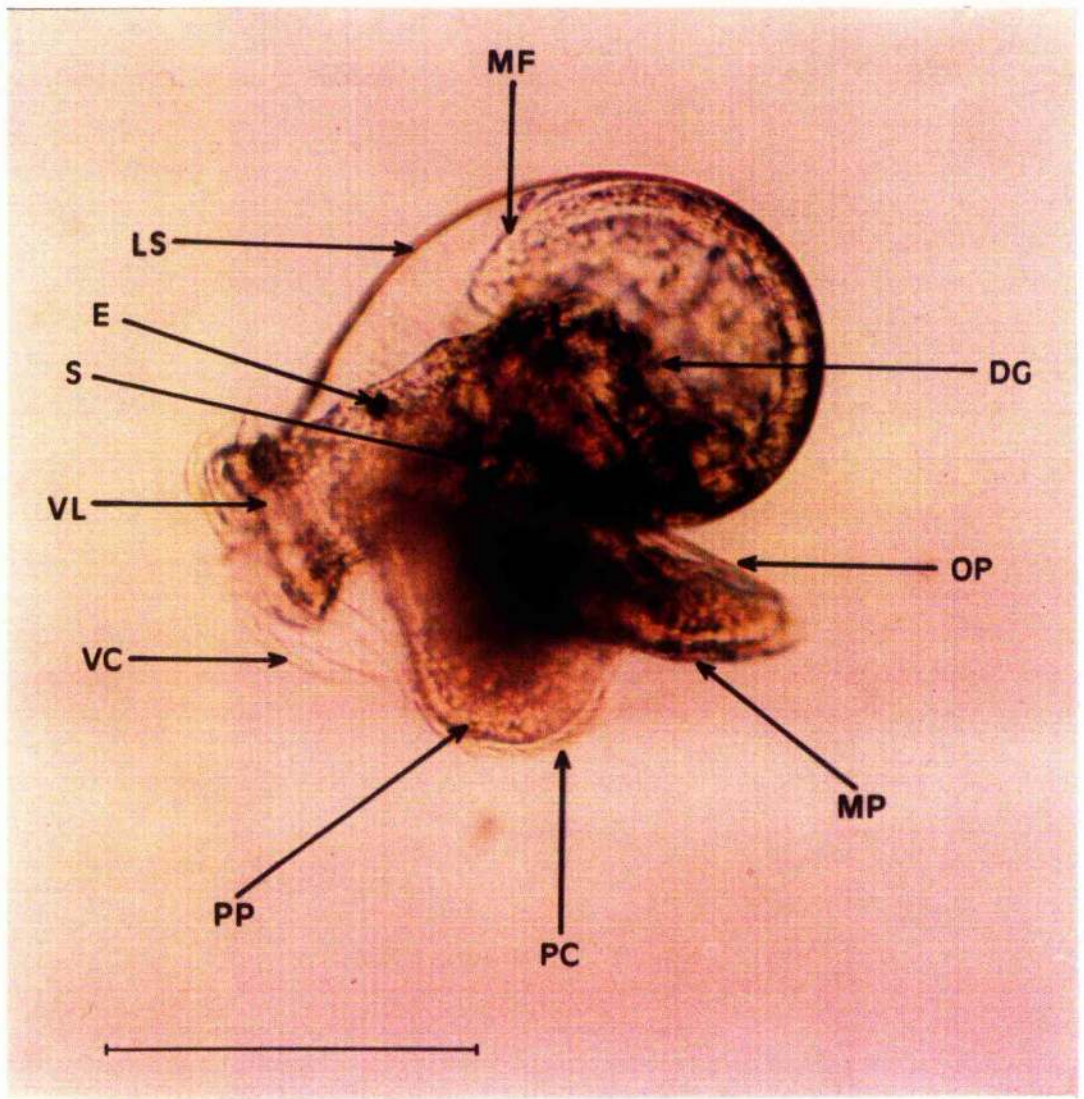
The larval shell (LS) and extended velar lobes (VL) with attached velar cilia (VC) of the swimming larva are clearly visible. The larva is capable of retracting the cephalopodal mass back into the shell at will. To achieve this the velar cilia cease beating, allowing the velar lobes to withdraw and the operculum (OP), which is seen here extended, is brought back by the larval retractor muscle to cover the shell cavity. On encountering a substratum, the larva may use both the propodium (PP) and propodial cilia (PC) to locate a suitable substratum and detect the metamorphic cue.

Scale bar represents 200µm.

Additional abbreviations used in the plate show:

E	eye
S	statocyst
MP	metapodium
MF	mantle fold
DG	digestive gland





**Plate 2.5a, b &c The Veliger Shell of *Adalaria proxima*.**

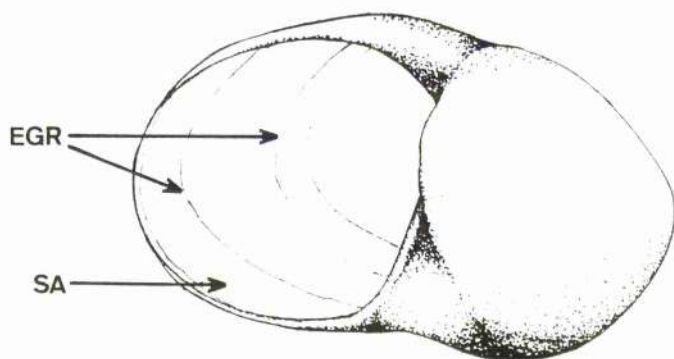
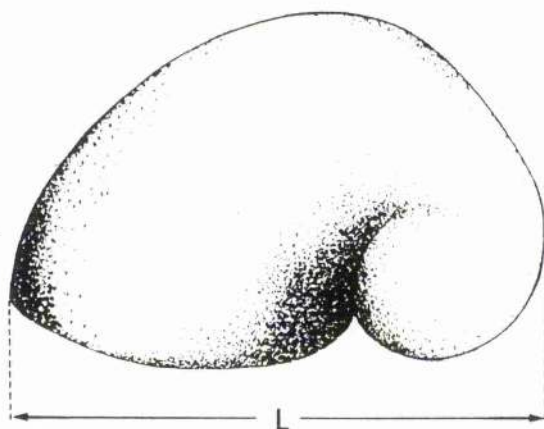
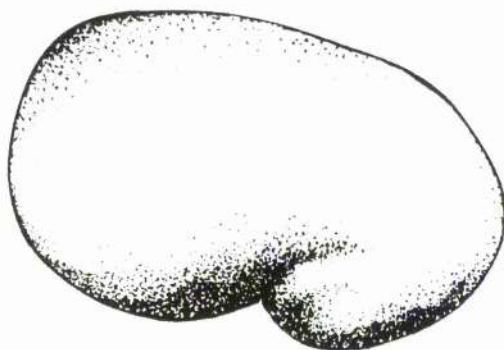
**a (above) Dorsal view**

**b (middle) Lateral view.** Shell length (L) was obtained using the maximum lateral shell dimension.

**c (below) Ventral view** (adapted from Thompson, 1958). Growth rings (EGR) on the shell are visible here through the shell aperture (SA) on the inside of the anterior-dorsal surface. No shell growth occurs in *A. proxima* during the pelagic larval stage (Thompson, 1958) - therefore any growth bands apparent on shells will be embryonic in origin.

The larval shell *in vivo* is illustrated in Plate 2.3.

Scale bar represents 200µm.



twice filtered (0.45 $\mu$ m, thence 0.2 $\mu$ m) sea water, hereafter referred to as TFSW (adapted from Smaldon & Lee, 1979). The morphology of the veliger shell, which does not grow during the pelagic larval phase (Thompson, 1958), and the method of length measurement are illustrated in Plates 2.5a, b & c. Upon hatching, the shells of 20 veliger larvae from selected spawn masses were measured using a Wild-E binocular microscope equipped with an ocular micrometer resulting in an accuracy of 4.47 $\mu$ m. The shells of a total of 26 spawn masses from five populations (Cuan Ferry, Argyll; Loch Eriboll, Sutherland; Robin Hood's Bay, North Yorkshire; Kinkell Braes, Fife, and Portaferry, Northern Ireland) of *Adalaria proxima* were measured. All spawn masses selected for shell length analysis were the first to be laid by an individual in order to facilitate the direct comparison of shell length both among and between populations.

### Data Analysis

Statistical analysis was performed using the Minitab © Release 8.1 Statistical Software package unless otherwise stated. The level of significance employed throughout the study was  $P < 0.05$ .

#### i. Zygote Diameter & Number

Exploratory data analysis confirmed that egg sizes within spawn masses were normally distributed. Normality was initially tested graphically using normal probability plots and then confirmed for each of the spawn masses using the Minitab correlation test for normality, considered equivalent to the Shapiro-Wilk test for normality (Minitab Release 7 Handbook). Consequently, parametric statistical analysis was thereafter employed. All statistical analysis was performed upon egg diameter although egg size is expressed in volumetric terms in all figures and tables. Egg diameter was considered appropriate for analysis, being a linear measure, whilst egg volume was employed for later discussion, the latter being considered a more functional reflection of 'egg size', because considerable differences in egg volume are conferred by even small differences in egg diameter. The unbalanced nature of the both the egg diameter and number data required the use of unbalanced hierarchical analysis of variance (HANOVA) within the Genstat 5 Release 3.1 statistical package. Analysis of covariance (ANCOVA) for unbalanced data was performed by employing the general linear model (GLM) within Minitab. Where ANOVA established a significant difference in the egg sizes

of first-laid spawn masses, a *posteriori* multiple pairwise comparison by Tukey's HSD was employed to identify the significant differences in mean values.

## ii. Embryonic Period

Embryonic development data were treated in the same manner as detailed above using HANOVA (Genstat 5 Release 3.1). The relationship between embryonic period and water temperature was investigated using daily tank temperature data obtained from the mean reading of three mercury thermometers permanently mounted within the adult culture tanks. Temperature data spanned a 110d period from 16<sup>th</sup> January (28d prior to the start of spawning) to 5<sup>th</sup> May 1993 (the last day on which spawning occurred). The distribution of water temperature data was confirmed to be normal following application of the Minitab normality correlation test (previously addressed above). Investigation of the duration of embryonic period as a function of selected variables was performed using Model I or Model II linear regression. Model II regression is considered appropriate when both the X and Y values are prone to natural variation and when X values are not fixed and are liable to measurement error. As a consequence, Model I regression was employed in investigating the relationship between embryonic period and number of the spawn mass in the laying succession, whilst analysis of both embryonic period on egg diameter and embryonic period on temperature data was considered to require Model II regression. Analysis of covariance (ANCOVA) was performed for unbalanced data sets by using the general linear model (GLM) method.

## iii. Hatching Success

Percentage data obtained from hatching success observations were arc-sine transformed before analysis by ANOVA (Genstat 5 Release 3.1). Levels of hatching success are expressed in the text, figures and tables as the back-transformed angular mean values and are displayed with the associated standard errors where appropriate.

## iv. Larval Shell Size

Larval shell length data were analysed using one-way ANOVA. Model II regression analysis was used to investigate the relationship between zygote diameter and larval shell length.



## 2.3 RESULTS

### Variation in Egg Size At The Intra- And Interpopulation Levels

The mean values of egg volume for the eight United Kingdom populations of *Adalaria proxima* are displayed in Table 2.1a. The mean egg diameter for 259 spawn masses in total was determined. The resultant overall population mean egg volumes included all spawn masses, irrespective of position in the spawn sequence, and were derived from a maximum of 62 and a minimum of 10 constituent spawn masses. Overall population means ranged from a minimum of  $2.15 \cdot 10^{-3} \text{mm}^3$  (diameter  $160.15 \mu\text{m}$ ) from Portaferry up to a maximum of  $3.10 \cdot 10^{-3} \text{mm}^3$  (corresponding to a diameter of  $180.92 \mu\text{m}$ ) from Menai Bridge, North Wales. Individual *A. proxima* adults were observed to lay between one and eleven spawn masses before their senescence and death.

It became apparent during the course of the study that the typical lengths of spawn mass sequences differed between sites; Kinkell Braes, for example, had a maximum spawn sequence of only four, whilst individuals from both Cuan Ferry and Portaferry laid up to eleven. Therefore, in order to facilitate direct comparison between populations, the mean egg size of first-laid spawn masses was determined for each population. Table 2.1a shows that the population mean egg sizes derived exclusively from first-laid spawn mass data were, for every population, greater than the means obtained by including all subsequent spawn masses laid by individuals within a population. The largest eggs for first-laid spawn masses were again produced by Menai Bridge animals ( $3.276 \cdot 10^{-3} \text{mm}^3$ ) whilst the smallest were laid by individuals from Portaferry ( $2.269 \cdot 10^{-3} \text{mm}^3$ ). One-way ANOVA (see Table 2.1b) showed there to be a significant difference between mean population egg volumes of first-laid spawn masses ( $F_{7,73} = 13.37$ ,  $P < 0.001$ ). *A posteriori* multiple pairwise comparison by Tukey's HSD, the results of which are illustrated in Figure 2.2a, identified these significant differences between populations. It is apparent from the Tukey groupings (denoted by superscript type above the appropriate columns) that the mean population egg sizes of first-laid spawn masses are not bimodally distributed, but rather that a range of

**Table 2.1a (above). Mean Values of Zygote Diameter and Corresponding Egg Volume For Eight United Kingdom Populations of *Adalaria proxima*.** The typical length of spawn sequences differed between populations. Therefore to allow direct comparisons across populations the mean egg size of first spawn masses laid by individuals was determined. The geographic locations of each population are illustrated in Plate 2.1. The number of spawn masses composing the appropriate mean values are denoted by 'n'.

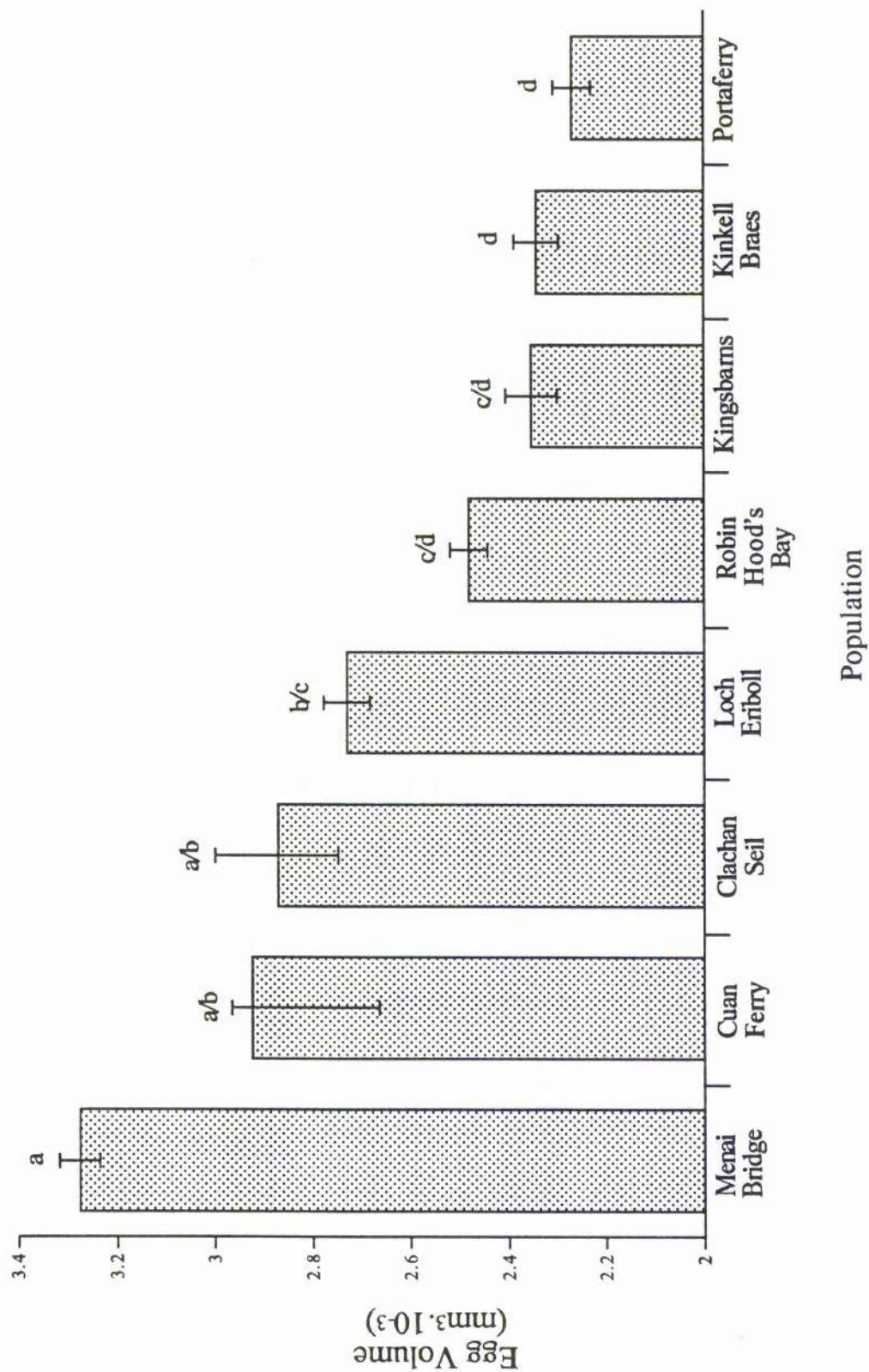
**Table 2.1b (below). ANOVA Of The Mean Egg Volume Of First-laid Spawn Masses For All Eight Populations Displayed In Table 2.1b. (above).** *A posteriori* multiple comparisons identifying the significant differences between the egg size of first-laid spawn masses are displayed in Figure 2.2a.

Population	Mean Egg Sizes (First Spawn Masses)			Grand Mean Egg Sizes (All Spawn Masses)		
	n	Diameter $\mu\text{m}$	Volume $\text{mm}^3 10^{-3}$	n	Diameter $\mu\text{m}$	Volume $\text{mm}^3 10^{-3}$
Menai Bridge, Wales	6	184.26	3.276	40	180.92	3.101
Cuan Ferry, Argyll	13	177.42	2.924	46	172.89	2.706
Clachan Seil, Argyll	10	176.36	2.872	10	176.36	2.872
Loch Eriboll, Sutherland	15	173.39	2.729	32	170.24	2.582
Robin Hood's Bay, England	12	167.96	2.481	35	163.73	2.298
Kingsbarns, Fife	10	165.02	2.353	10	165.02	2.353
Kinkell Braes, Fife	4	164.79	2.343	14	163.6	2.293
Portaferry, N. Ireland	11	163.03	2.269	62	160.15	2.151

	df	SS	MS	Fvalue	P
<i>Mean Egg Volume</i>					
between populations	7	3261.8	466.0	13.37	<0.001*
within populations	73	2543.4	34.8		



**Figure 2.2a. Mean Egg Volume Of First-Laid Spawn Masses From Eight United Kingdom Populations of *Adalaria proxima*.** Superscript type denotes groupings resulting from a *posteriori* multiple comparison by Tukey's HSD. Groupings are considered significantly different at the  $P < 0.05$  significance level. The corresponding absolute values and sample sizes are presented in Table 2.1a. Individuals were collected as adults from Clachan Seil and Kingsbarns two months before spawning commenced whilst individuals were collected from all other sites a few weeks after settling and were raised to maturity in the laboratory.



sizes is present. It is notable that the U.K. east coast sites (Kinkell Braes and Kingsbarns, which are within 11 km of each other) and Robin Hood's Bay possess egg sizes which lie within the same Tukey grouping. Such also is the case for Clachan Seil and Cuan Ferry (mean volumes of  $2.87 \cdot 10^{-3} \text{mm}^3$  and  $2.924 \cdot 10^{-3} \text{mm}^3$  respectively) which are also geographically proximate to each other. This, however, constitutes the only geographic trend in mean population egg size evident from the data. Plate 2.1, which shows the locations of each survey site, when considered in concert with the pattern of mean population egg volumes displayed in Figure 2.2a, offers no evidence for the existence of a latitudinal cline in egg size between populations over the range considered.

The basis for such significant interpopulational differences in egg size was investigated by examining the sources of such variance both within and between populations. Egg size distribution was therefore investigated across six sites - Menai Bridge, Cuan Ferry, Loch Eriboll, Robin Hood's Bay, Kinkell Braes and Portaferry. Egg volume was examined as a function of site, and of both individual adult and position of the spawn mass within an individual's spawning sequence (the mean values of sequential spawn mass egg size, accompanied by the number of spawn masses comprising the appropriate mean, are displayed in Table 2.1c). Analysis of the data required unbalanced hierarchical ANOVA (HANOVA). The lowest level of the hierarchy was represented by between-individual variation in egg diameter. Inclusion of within-individual variation in egg size with spawn sequence was precluded due to inadequate replication - the mean egg size of each spawn mass was denoted by only one integer. Therefore, HANOVA was first performed examining variance attributable to site and to individual adult, with multiple spawn masses laid by individuals being considered replicates (see Table 2.1d). A highly significant proportion of the total variance was found to be attributable to the effect of population ( $P < 0.001$ ). In contrast, variation between individuals nested within populations was established to be non-significant ( $P = 0.130$ ). In addition, Table 2.1e shows spawn sequence to exert a significant effect upon egg size ( $P < 0.01$ ). It is notable that the variation in egg size accounted for at the between-population level is considerably greater than that between sequential spawn masses (this is apparent on comparison of the relative Mean Square and Variance ratios in Table 2.1e).

Because HANOVA established there to be no significant variance in egg size at the between-individual level, the effect of spawn sequence across

**Table 2.1c. Mean Egg Volume Within Spawn Sequences For Six United Kingdom Populations of *Adalaria proxima*.** Integers in parentheses represent the number of spawn masses composing the appropriate mean. Adults from different populations did not lay the same number of spawn masses and therefore groups where no spawn masses were available are denoted by dashed lines. Also some spawn could not be measured because cleavage had commenced.

Spawn Mass Laying Order	Mean Egg Volume (10 <sup>-3</sup> mm <sup>3</sup> )					
	Menai Bridge, Wales	Cuan Ferry, Argyll	Loch Eriboll, Sutherland	Robin Hood's Bay, N. England	Kinkell Braes, Fife	Portaferry, N. Ireland
1 <sup>st</sup>	3.276 (6)	2.924 (13)	2.729 (15)	2.481 (12)	2.343 (4)	2.268 (11)
2 <sup>nd</sup>	3.153 (5)	2.644 (6)	2.375 (8)	2.277 (9)	2.337 (6)	2.279 (5)
3 <sup>rd</sup>	3.062 (6)	2.575 (6)	2.515 (6)	2.193 (8)	2.242 (3)	1.949 (5)
4 <sup>th</sup>	2.980 (6)	2.803 (3)	2.750 (1)	2.174 (4)	1.989 (1)	2.319 (8)
5 <sup>th</sup>	2.863 (3)	2.680 (6)	2.534 (1)	2.021 (2)	-	2.219 (9)
6 <sup>th</sup>	3.041 (2)	2.483 (5)	2.435 (1)	-	-	2.152 (6)
7 <sup>th</sup>	2.761 (2)	2.768 (3)	-	-	-	2.097 (3)
8 <sup>th</sup>	-	2.560 (3)	-	-	-	1.921 (2)
9 <sup>th</sup>	-	-	-	-	-	2.039 (2)
11 <sup>th</sup>	-	2.504 (1)	-	-	-	2.006 (1)

**Table 2.1d (top). Hierarchical ANOVA (HANOVA) Examining The Effect of Population And Individual On Mean Egg Volume.** The analysis concerned the reproductive output of six laboratory reared populations - Menai Bridge (n=30 spawn masses), Cuan Ferry (n=46), Loch Eriboll (n=32), Robin Hood's Bay (n=35), Kinkell Braes (n=14), and Portaferry (n=52). Spawn masses were nested within individuals and individuals were, in turn, nested within populations. Inclusion of the effect of spawn sequence within this analysis was precluded by inadequate replication at the lowest hierarchical level (each spawn mass was represented by only one mean value). Asterisks denote results considered to be significant (at the  $P < 0.05$  level).

**Table 2.1e (middle). HANOVA Examining The Effect of Population And Spawn Sequence On Mean Egg Volume.** (Data set as above). Both population and spawn sequence appeared to exert a significant effect upon egg size. It is notable that a considerable majority of the observed variance in egg size may be explained at the population, and not the spawn sequence, level (see the relative values of the mean-squares and variance ratios). Asterisks denote results considered to be significant (at the  $P < 0.05$  level).

**Table 2.1f (bottom). The Effect of Spawn Sequence On Egg Volume Across Populations.** HANOVA demonstrated spawn sequence to exert a significant effect on egg size (see Table 2.1e). The nonsignificant interaction term provided by ANCOVA (using a general linear model [GLM]) showed this effect to be uniform across all six populations ( $P = 0.263$ ). Asterisks denote results considered to be significant.

	df	SS	MS	Variance ratio	P
<i>Source</i>					
Between Population	5	9094	1818.8	45.18	<0.001*
Between Individual	79	3180	40.3	1.25	0.130
Within Individual	124	3991	32.2		
Total	208	16265	78.2		

	df	SS	MS	Variance ratio	P
<i>Source</i>					
Between Population	5	9094	1818.8	30.02	<0.001*
Between Spawn Sequence	35	2120	60.6	2.01	<0.01*
Within Spawn Sequence	168	5051	30.1		
Total	208	16265	78.2		

	df	SS	MS	FValue	P
<i>Source</i>					
Between Population	5	9093.8	491.3	15.87	<0.001*
Between Sequence	1	870.1	545.15	17.61	<0.001*
Population * Sequence	5	202.0	40.4	1.30	0.263
Error	197	6099.4	30.96		
Total	208	16265.3			



populations was then evaluated using ANCOVA (by GLM). The absence of a significant interactive term ( $P=0.263$ , see Table 2.1f) indicated the effect of spawn sequence upon egg size to be uniform across all six populations. Table 2.1c shows that (with the exception of spawn masses from the Loch Eriboll population) the larger mean egg sizes tend to occur within the earlier laid spawn masses whilst the minimum egg sizes were observed towards the end of the spawning sequence. This is illustrated graphically for each population in Figures 2.2b-g. Indeed, in four of the six populations the maximum mean egg volume is represented by the first spawn masses to be laid. For example, the first laid spawn masses resulting from the Menai Bridge population possess the largest mean egg volume ( $3.28 \cdot 10^{-3} \text{ mm}^3$ ) evident within this population, whilst the smallest eggs of  $2.76 \cdot 10^{-3} \text{ mm}^3$  are present in the 7th laid spawn masses, which were the last to be laid within the Menai Bridge population.

#### Variation in Spawn Mass Egg Numbers at the Intra- and Interpopulation Levels

The numbers of eggs contained in a total of 209 spawn masses were determined in the course of the present study. The present study employed direct determination of egg numbers in each spawn mass. The density of egg distribution within the spawn mass stroma was observed to be non-uniform both within and between populations. Whilst the greater proportion of this variation appeared unpredictable, spawn masses produced late in an individual's reproductive period were frequently observed to exhibit a reduced density of egg distribution relative to earlier laid spawn masses. It is worthy of note therefore that the 'size' of the spawn mass as indicated by damp weight or by stroma length may not necessarily be a reliable reflection of egg number where the density of egg distribution varies in an unforeseeable manner.

The mean number of eggs per spawn mass for six populations of *Adalaria proxima* are displayed at the foot of Table 2.2a. The mean number of eggs within spawn masses (illustrated in Figure 2.3a) varied markedly from a maximum of 1792 eggs in spawn masses resulting from the Loch Eriboll population down to a minimum of 810 in spawn masses from the Portaferry population. The origin of such interpopulational differences in spawn mass size (mean number of eggs) was investigated by examining possible sources of variation within populations, that is, the effect of the individual and position in the spawn sequence. In a manner

**Figures 2.2b-g. Mean Egg Volume for Spawn Masses Laid Sequentially through the Spawning season.** The corresponding absolute values and sample sizes are presented in Table 2.1c.

Figure 2.2b. Menai Bridge, Wales.

Figure 2.2c. Cuan Ferry, Argyll.

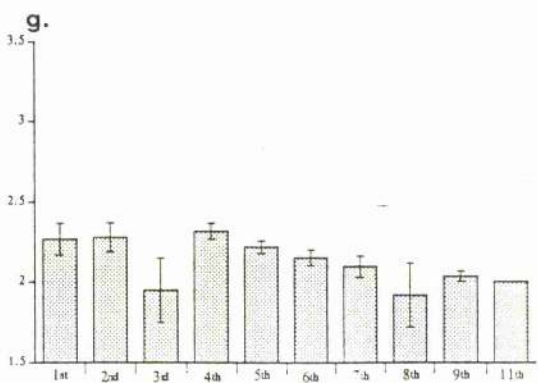
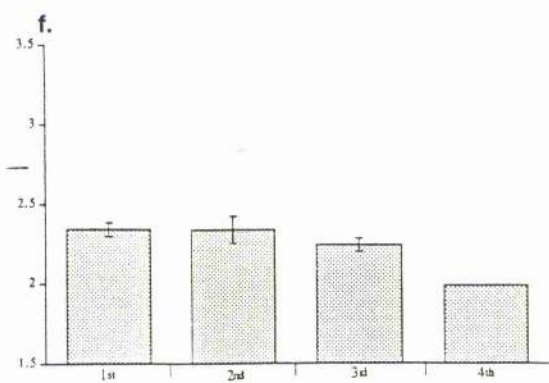
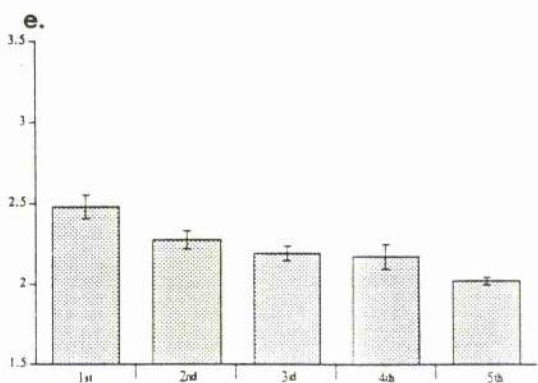
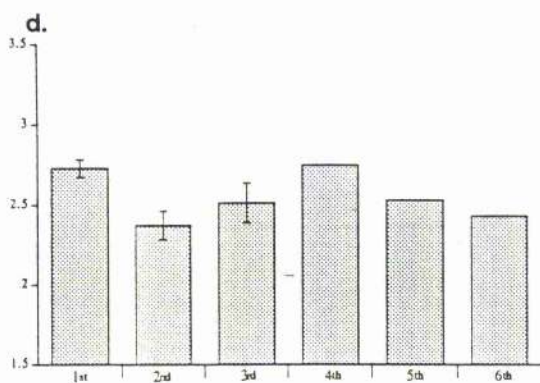
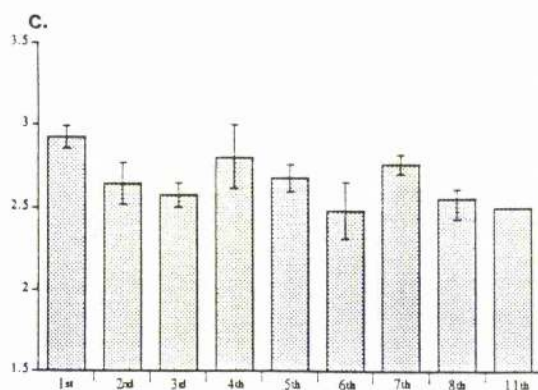
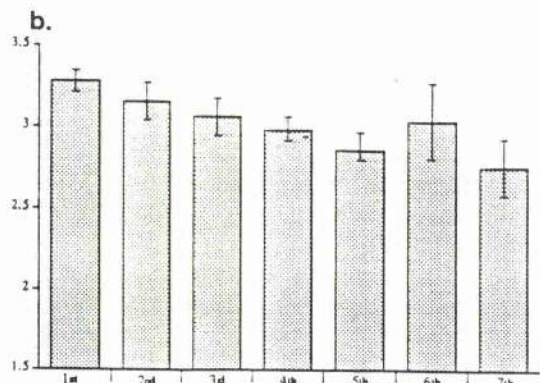
Figure 2.2d. Loch Eriboll, Sutherland.

Figure 2.2e. Robin Hood's Bay, N.England.

Figure 2.2f. Kinkell Braes, Fife.

Figure 2.2g. Portaferry, N.Ireland.

Mean Egg Volume ( $10^{-3}\text{mm}^3$ )



Spawn Sequence

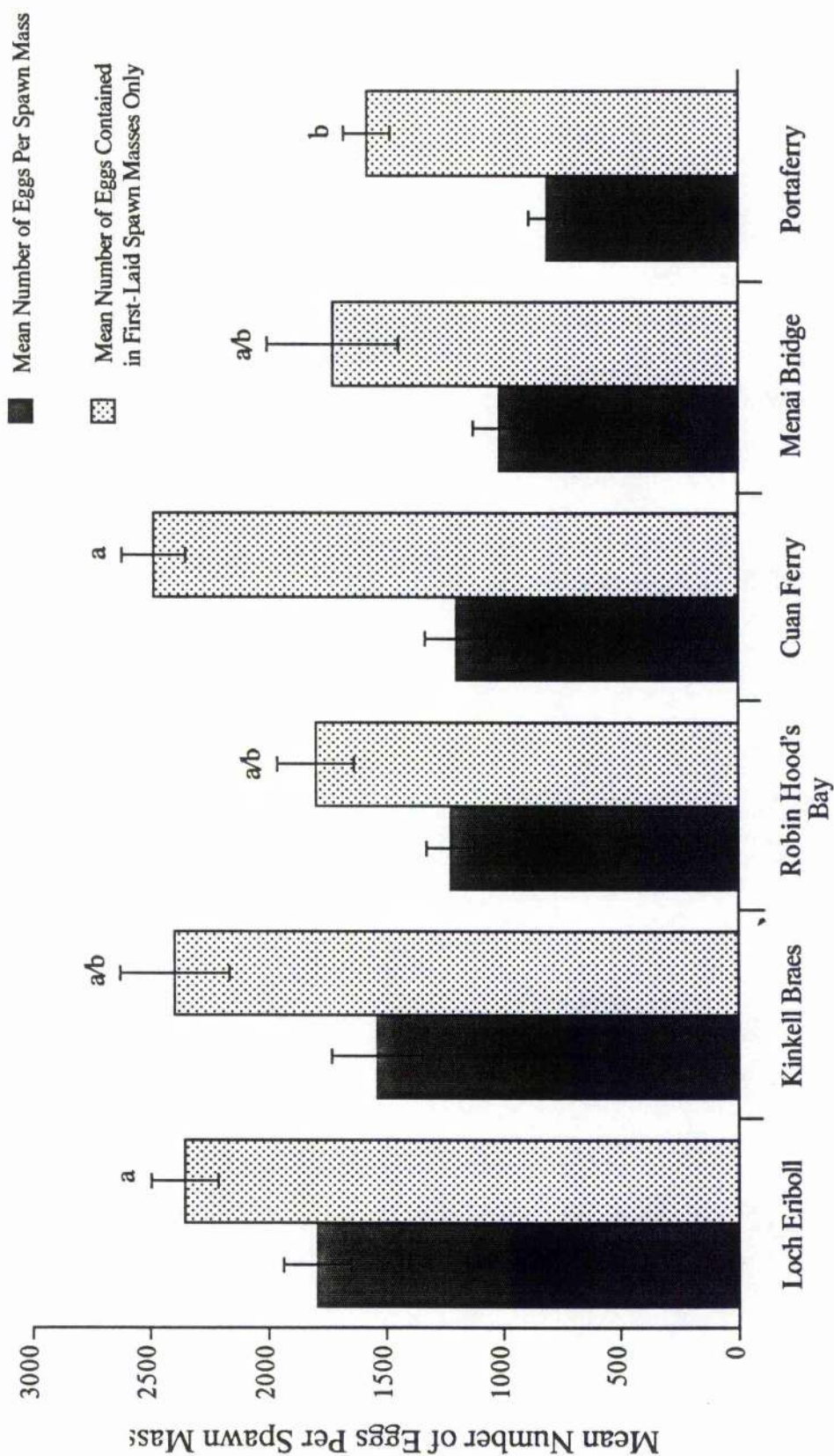
**Table 2.2a. The Mean Number Eggs within Sequentially Laid Spawn Masses for Six U.K. Populations of *Adalaria proxima*.** Population grand means (egg numbers per spawn mass) are displayed at the bottom of each column. Populations are presented within this table ranked in descending magnitude of egg numbers per spawn mass. Integers in parentheses represent the number of spawn masses composing the appropriate mean. Adults from different populations did not lay the same number of spawn masses and therefore groups where no spawn masses were available are denoted by dashed lines.

# Mean Number of Eggs Per Spawn Mass

Spawn Sequence	Loch Eriboll, Sutherland	Kinkell Braes, Fife	Robin Hood's Bay, N. England	Cuan Ferry, Argyll	Menai Bridge, Wales	Portaferry, N. Ireland
1st	2353 (15)	2394 (4)	1796 (12)	2481 (13)	1723 (6)	1577 (11)
2nd	1750 (8)1	1477 (6)	1073 (9)	897 (6)	570 (5)	1013 (5)
3rd	997 (6)	852 (3)	953 (8)	945 (6)	793 (6)	860 (5)
4th	1012 (1)	521 (1)	732 (4)	581 (3)	726 (6)	845 (8)
5th	657 (1)	-	532 (2)	700 (6)	525 (3)	460 (9)
6th	396 (1)	-	-	412 (5)	343 (2)	366 (6)
7th	-	-	-	668 (3)	415 (2)	427 (3)
8th	-	-	-	563 (3)	-	242 (2)
9th	-	-	-	-	-	172 (2)
11th	-	-	-	-	154 (1)	215 (1)
Population Mean	1792 (32)	1537 (14)	1224 (35)	1199 (46)	1013 (30)	810 (52)

**Figure 2.3a.** Grand Mean Number of Eggs Per Spawn Mass for Six United Kingdom Populations of *Adalaria proxima*. The mean number of eggs contained in first-laid spawn masses and the mean number of eggs contained in all spawn masses are shown for each population. One-way ANOVA established there to be a significant difference in the mean number of eggs contained by these first-laid spawn masses between populations ( $F_{5,56}=3.88$ ,  $P<0.05$ ) and the relevant Tukey groupings are denoted by uppercase superscript characters above the appropriate columns. The corresponding absolute values and number of spawn masses comprising the relevant mean values are shown in Table 2.2a.







similar to the previous analysis of egg volume, the nature of the data necessitated the use of HANOVA, with the lowest level of the hierarchy represented by variation at the between-individual level. Table 2.2b shows the level of variation in spawn mass size attributable to individuals within populations to be nonsignificant ( $P>0.05$ ) when compared to the proportion of variation in spawn mass size attributable to the population effect ( $P<0.001$ ).

Spawn masses were classed within populations according to their position in each adult's laying sequence (e.g. first-laid, second-laid etc. to a maximum of 11th-laid). The resultant mean values for each population are shown in Table 2.2a. It is apparent from Table 2.2a that adults both within and between populations did not lay a uniform number of spawn masses over the course of this study. Some adults within populations laid as many as 11, as was the case within the Portaferry and Menai Bridge populations (see Table 2.2a), whilst in the Kinkell Braes population a maximum sequence of just four spawn masses was laid. Examination of the proportion of observed variance in spawn mass size attributable to spawn sequence within populations and that attributable at the between-population level was performed by HANOVA. The spawn sequence of spawn masses was found to account for a highly significant proportion of the variance ( $P<0.001$ ). It is notable that the degree of observed variance attributable to spawn sequence was so great as to render the effect of population nonsignificant in comparison ( $P>0.05$ ). The marked decline in mean egg numbers with increasing position in the spawn sequence is illustrated for each population in Figures 2.3b-g. The relative reduction in egg numbers between the first and last-laid spawn masses differs between populations by only 16%. The greatest decreases in mean egg number through the spawning sequence are, for example, apparent in the Portaferry (86.4%) and Loch Eriboll (83.2%) populations, whilst Robin Hood's Bay shows the minimum decrease (70.4%), with Kinkell Braes, Cuan Ferry and Menai Bridge being intermediate (78.2%, 77.3%, and 75.9% respectively).

It is clear from Table 2.2a that the first-laid spawn masses contained a greater number of eggs than did all subsequent spawn masses for all populations, and further investigation of their absolute magnitude and relative contribution to total reproductive output was therefore considered important. The egg numbers of first-laid spawn masses ranged from a maximum of 2481 in the Cuan Ferry population down to a minimum of 1577 in the Portaferry population (see Table

**Table 2.2b (above). Hierarchical ANOVA (HANOVA) Examining The Effect of Population And Individual On Mean Spawn Mass Egg Number.** The analysis contained the reproductive output of six populations - Menai Bridge (n=30 spawn masses), Cuan Ferry (n=46), Loch Eriboll (n=32), Robin Hood's Bay (n=35), Kinkell Braes (n=14), and Portaferry (n=52). Inclusion of the effect of spawn sequence within this analysis was precluded by inadequate replication at the lowest hierarchical level (each spawn mass was represented by only one mean value). The relevant absolute values of mean number of eggs per span mass are displayed in Table 2.2a. Asterisks denote results considered to be significant (at the  $P < 0.05$  level).

**Table 2.2c (below). HANOVA Examining The Effect of Population And Spawn Sequence On Mean Spawn Mass Egg Number.** Asterisks denote results considered to be significant. (Data set as above).

	df	SS	MS	Variance ratio	P
<i>Source</i>					
Between Population	5	21745558	4349112	10.99	<0.001*
Between Individual	79	31270162	395825	0.69	>0.25
Within Individual	124	70824872	571168	-	-
Total	208	123840592	595387	-	-

	df	SS	MS	Variance ratio	P
<i>Source</i>					
Between Population	5	21745556	434911	2.04	>0.05
Between Spawn Sequence	35	74509296	2128837	12.96	<0.001*
Within Spawn Sequence	168	27585744	164201	-	-
Total	208	123840592	595387	-	-

**Figures 2.3b-g. Mean Egg Numbers of Sequentially Laid Spawn Masses.** Error bars denote the appropriate standard error about the mean. Absolute values and the sample sizes from which the means are derived are shown in Table 2.2a.

**Figure 2.3b.** Loch Eriboll, Sutherland.

**Figure 2.3c** Kinkell Braes, Fife.

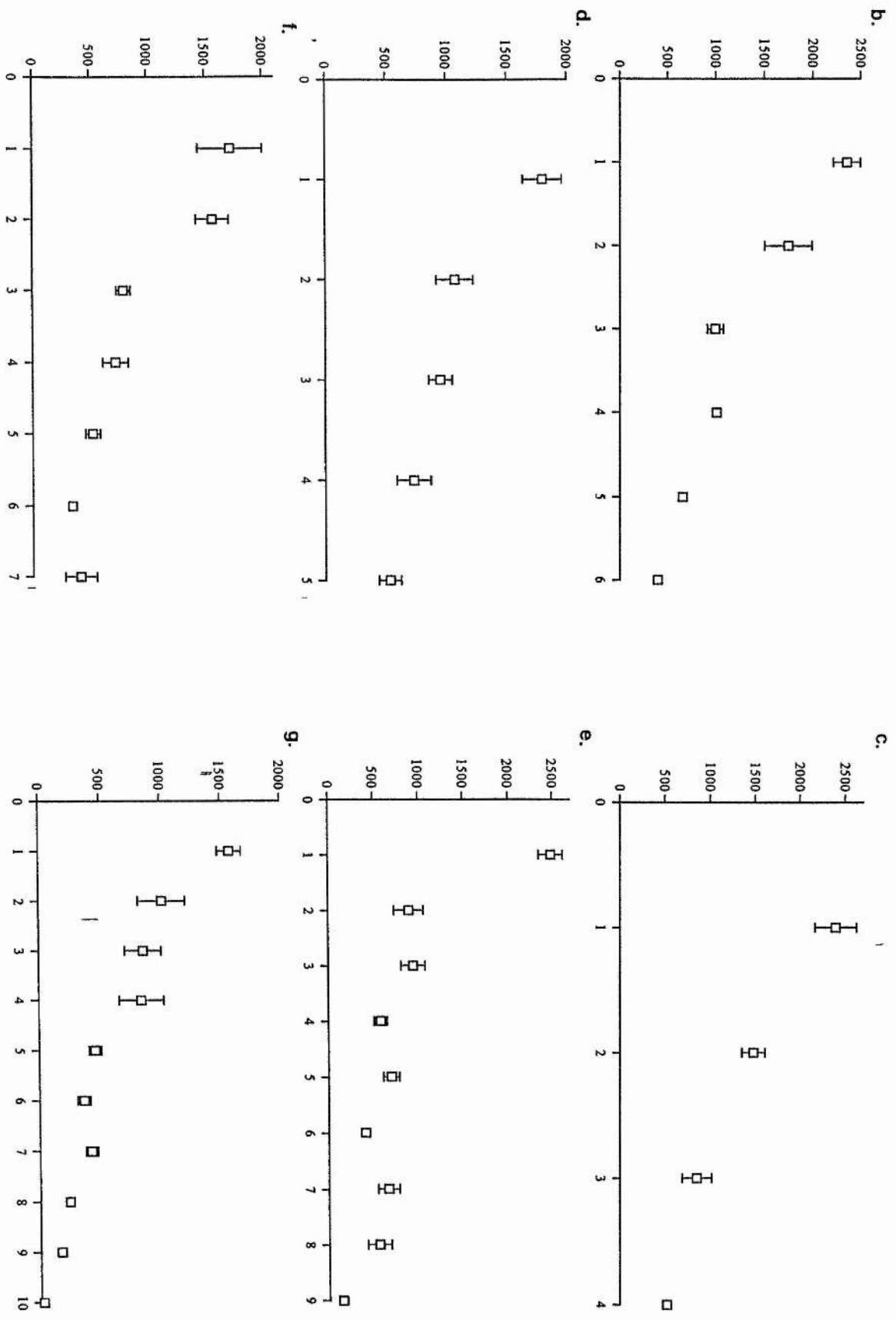
**Figure 2.3d** Robin Hood's Bay.

**Figure 2.3e** Cuan Ferry, Argyll.

**Figure 2.3f** Menai Bridge, Wales.

**Figure 2.3g** Portaferry, N.Ireland.

# Number of Eggs Per Spawn Mass



Spawn Sequence

2.2a). One-way ANOVA established there to be a significant difference between populations in the number of eggs contained within these first-laid spawn masses ( $F_{5,56}=3.88$ ,  $P<0.05$ ). Figure 2.3a illustrates that in all populations the first-laid spawn masses contained significantly more eggs than did the corresponding population means (derived from all spawn masses irrespective of laying order). Subsequent *a posteriori* multiple comparison established that both Loch Eriboll and Cuan Ferry individuals produced first-laid spawn masses containing significantly more eggs than did those from Portaferry (2353, 2394 and 1577 respectively). Spawn masses from the remaining three locations (Kinkell Braes [2394], Robin Hood's Bay [1796] and Menai Bridge [1723]) were considered to contain an intermediate number. It is notable from Figure 2.3a that there exists no apparent trend between the number of eggs contained within first-laid spawn masses and the mean number of eggs contained within all spawn masses for each population. The proportion of the total reproductive output invested within the first-laid spawn mass was thus considered further. It is apparent from Table 2.2d that adults from Portaferry invest the lowest proportion of total egg production in the first-laid spawn masses (25.5%) relative to conspecifics from other populations. In contrast, first-laid spawn masses from Kinkell Braes constitute a mean of 45.7 % of the total egg production. Table 2.2d also provides an indication of the relative distribution of reproductive effort in spawn mass laid over the course of the reproductive period. There is, as expected, a strong trend of decreasing relative proportion of total egg production invested in later sequential spawn masses evident in all populations.

#### The Relationship Between Egg Size and Egg Number

The relationship between number and size of eggs within spawn masses was investigated by considering mean zygote diameter as a function of egg number. The results of regression analysis performed upon a total of 209 spawn masses within six populations are displayed in Table 2.2e. No significant relationship was established between mean zygote diameter and egg number within spawn masses originating from Loch Eriboll ( $F_{1,30}=0.98$ ,  $P=0.329$ ), Kinkell Braes ( $F_{1,12}=2.11$ ,  $P=0.172$ ) and Portaferry ( $F_{1,50}=0.26$ ,  $P=0.614$ ), the relevant regression plots are illustrated in Figures 2.3h, 2.3i and 2.3m respectively. Conversely, a significant positive linear relationship spawn mass egg number and



**Table 2.2d. The Relative Contribution of Sequentially Laid Spawn Masses to the Total Mean Number of Eggs Produced Within Each Population.** The values presented within this table correspond to absolute mean egg numbers per spawn mass presented in Table 2.2a. Populations are presented within this table ranked in the same manner as in Table 2.2a. in descending magnitude of absolute egg numbers per spawn mass. Proportions are expressed within the table as percentages. Integers in parentheses represent the number of spawn masses composing the appropriate mean. Adults from different populations did not lay the same number of spawn masses and groups where no data were available are denoted by dashed lines.

Contribution of Sequential Spawn Mass Groups to the Total Mean Number  
of Eggs Produced Within Each Population  
(%)

Spawn Mass Laying Order	Loch Eriboll, Sutherland	Kinkell Braes, Fife	Robin Hood's Bay, N. England	Cuan Ferry, Argyll	Menai Bridge, Wales	Portaferry, N. Ireland
1 <sup>st</sup>	32.8 (15)	45.7 (4)	35.3 (12)	34.2 (13)	32.8 (6)	25.5 (11)
2 <sup>nd</sup>	24.4 (8)	28.2 (6)	21.1 (9)	12.4 (6)	10.9 (5)	16.4 (5)
3 <sup>rd</sup>	13.9 (6)	16.2 (3)	18.7 (8)	13.0 (6)	15.2 (6)	13.9 (5)
4 <sup>th</sup>	14.2 (1)	9.9 (1)	14.4 (4)	8.1 (3)	13.8 (6)	13.7 (8)
5 <sup>th</sup>	9.2 (1)	-	10.5 (2)	9.7 (6)	10.0 (3)	7.6 (9)
6 <sup>th</sup>	5.5 (1)	-	-	5.6 (5)	6.5 (2)	6.0 (6)
7 <sup>th</sup>	-	-	-	9.2 (3)	7.9 (2)	6.9 (3)
8 <sup>th</sup>	-	-	-	7.8 (3)	-	3.9 (2)
9 <sup>th</sup>	-	-	-	-	-	2.9 (2)
11 <sup>th</sup>	-	-	-	-	2.93 (1)	3.5 (1)
Total	100	100	100	100	100	100

**Table 2.2c. Spawn Mass Mean Zygote Diameter As A Function Of  
Spawn Mass Egg Number For Six U.K. Populations.** The relevant  
regression plots for each population are illustrated in Figures 2.3h-m. Those  
populations in which a significant linear relationship was established are denoted  
by an asterisk.

Population	n	r Value	Regression Equation	<i>P</i> value
Loch Eriboll	32	0.178	No significant correlation	0.329
Kinkell Braes	14	0.387	No significant correlation	0.172
Robin Hood's Bay	35	0.339	$160 + 0.00300 \text{ Egg N}^2$	0.046*
Cuan Ferry	46	0.484	$169 + 0.00326 \text{ Egg N}^2$	0.001*
Menai Bridge	30	0.377	$177 + 0.00306 \text{ Egg N}^2$	0.040*
Portaferry	52	0.071	No significant correlation	0.614

**Figures 2.3h-m. Regression of Mean Zygote Diameter On Spawn Mass Egg Number.** The results of regression analysis are displayed in Table 2.2e.

**Figure 2.3h Loch Eriboll.** Regression ANOVA established there to be no significant linear relationship between spawn mass egg size and spawn mass embryonic period in this population.

**Figure 2.3i. Kinkell Braes.** Regression ANOVA established there to be no significant linear relationship between spawn mass egg size and spawn mass embryonic period in this population.

**Figure 2.3j Robin Hood's Bay Population.** Regression ANOVA established there to be a significant linear relationship between spawn mass egg size and spawn mass embryonic period in this population ( $F_{1,33}=4.31$ ,  $P<0.05$ ).

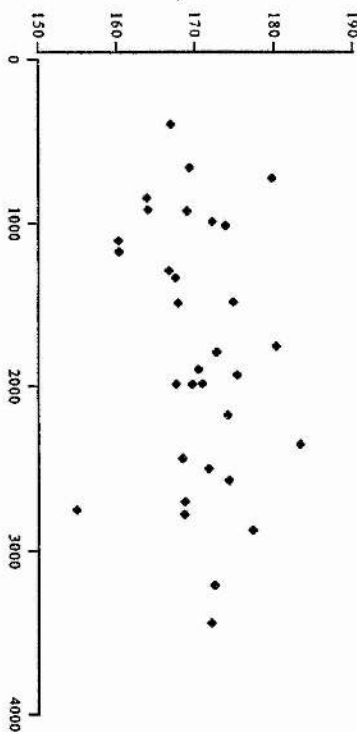
**Figure 2.3k Cuan Ferry.** Regression ANOVA established there to be a significant linear relationship between spawn mass egg size and spawn mass embryonic period in this population ( $F_{1,44}=13.52$ ,  $P<0.01$ ).

**Figure 2.3l Menai Bridge.** Regression ANOVA established there to be a significant linear relationship between spawn mass egg size and spawn mass embryonic period in this population ( $F_{1,28}=4.64$ ,  $P<0.05$ ).

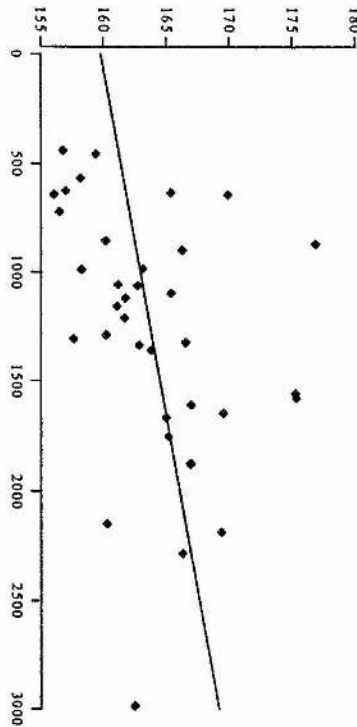
**Figure 2.3m Portaferry Population.** Regression ANOVA established there to be no significant linear relationship between spawn mass egg size and spawn mass embryonic period in this population (at the  $P<0.05$  level).

# Zygote Diameter ( $\mu\text{m}$ )

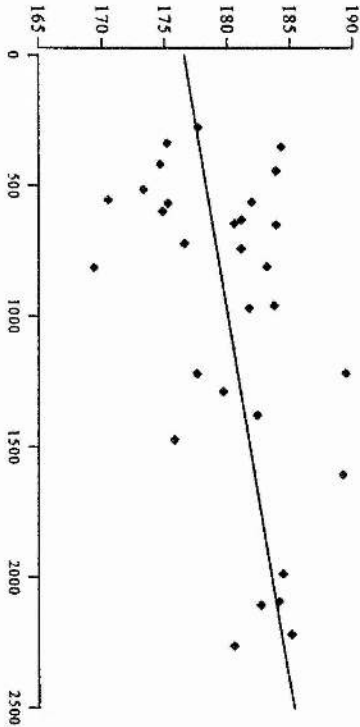
h.



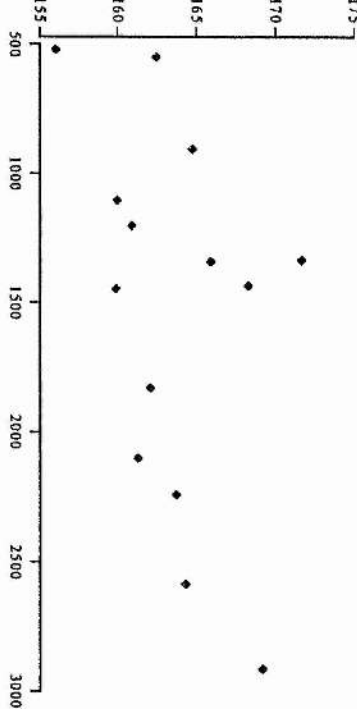
i.



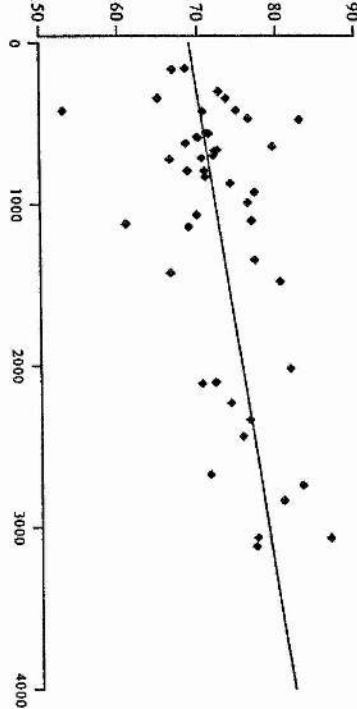
l.



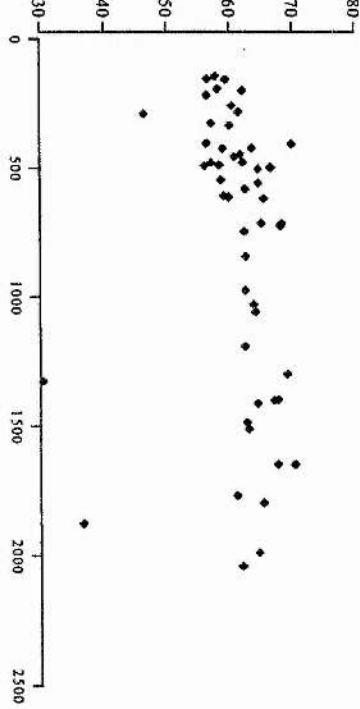
j.



k.



m.



Number of Eggs Per Spawn Mass



egg diameter was established within spawn masses associated with the remaining three populations - Robin Hood's Bay ( $F_{1,33}=4.31$ ,  $P<0.05$ ), Cuan Ferry ( $F_{1,44}=13.52$ ,  $P<0.01$ ) and Menai Bridge ( $F_{1,28}=4.64$ ,  $P<0.05$ ). The regression lines shown in Figures 2.3j, 2.3k, and 2.3l illustrate that within these three populations an increase in the mean number of eggs within each spawn mass is associated with an increase in the mean zygote diameter.

#### Variation in the Duration of the Intracapsular Embryonic Period at the Intra- and Interpopulation Levels

The intracapsular embryonic period constituted the time period (in days) from the laying of the spawn mass to the majority (>50%) of larvae hatching from that spawn mass at 6°C. A total of 188 spawn masses were monitored from six populations, and the resulting population mean intracapsular embryonic periods are displayed in Table 2.3a. There are several reasons why the numbers of observations from which mean population embryonic period was derived differ from those for which egg volume was determined. Eggs were not always available at zygote stage, and in cases where elongation or division of the zygotes had begun the measurement of egg diameter was precluded although the determination of embryonic period was not. In addition, spawn masses did not always develop successfully, and some were removed for experimental purposes before hatching. Therefore numbers may differ, although population mean embryonic periods are still derived from a considerable number of spawn mass embryonic periods ( $n=15$  to 40).

Population mean embryonic periods were observed to range from a minimum of 62.5d in progeny from Kinkell Braes up to a maximum of 71.0d for embryos from Cuan Ferry (see Table 2.3a). The cause of such significant differences in the embryonic developmental rate between populations was investigated by considering the presence of possible determining factors acting at the intrapopulation level.

##### **i. Variation in Intracapsular Embryonic Period Between Adults**

**Table 2.3a (top).** The Observed Spawning Seasons and Mean Intracapsular Embryonic Periods of *Adalaria proxima* Progeny from Kinkell Braes, Robin Hood's Bay, Loch Eriboll, Menai Bridge, Portaferry and Cuan Ferry. The observed spawning season spanned the period from the laying of the first spawn until the laying of the last within each population and contains only those spawn masses for which intracapsular embryonic period was determined. The intracapsular embryonic period was defined as the number of days from spawning (uncleaved zygotes) to larval hatching. The mean embryonic period (in days) for each population is displayed. The number of spawn masses composing the appropriate mean is denoted by 'n'.

**Table 2.3b (middle).** Hierarchical ANOVA (HANOVA) Examining The Effect of Population And Individual On Duration Of The Intracapsular Embryonic Period. Inclusion of the effect of spawn sequence within this analysis was precluded by inadequate replication at the lowest hierarchical level (each spawn mass was represented by only one mean value). Asterisks denote results considered to be significant (at the  $P < 0.05$  level).

**Table 2.3c (bottom).** HANOVA Examining The Effect of Population And Spawn Sequence On Duration Of The Intracapsular Embryonic Period. (Data set as above). Asterisks denote results considered to be significant.

Population	Observed Spawning Season		Grand Mean Embryonic Period	
	n		(days)	se
Cuan Ferry, Argyll	40	10th March - 5th May	71.0	1.0
Portaferry, N. Ireland	31	7th March - 29th April	68.0	1.0
Menai Bridge, Wales	31	13th February - 12th April	63.9	0.9
Loch Eriboll, Sutherland	35	8th March - 1st May	63.6	0.9
Robin Hood's Bay, N.England	36	10th March - 15th April	63.2	0.8
Kinkell Braes, Fife	15	13th March - 25th April	62.5	1.1

	df	SS	MS	Variance ratio	P
<i>Source</i>					
Between Population	5	1854	370.7	9.59	<0.001*
Between Individual	78	3016	38.7	1.46	<0.05*
Within Individual	104	2749	26.4	-	-
Total	187	7619	40.7	-	-

	df	SS	MS	Variance ratio	P
<i>Source</i>					
Between Population	5	185	370.7	8.88	<0.001*
Between Spawn Sequence	29	1211	41.8	1.40	>0.25
Within Spawn Sequence	153	4554	29.8	-	-
Total	187	7619	40.7	-	-

The number of individuals within each population varied from a minimum of five from Kinkell Braes (caused by high unexplained mortality prior to spawning), up to maximum of 17 adults from Cuan Ferry. In addition, the number of spawn masses laid by each individual was observed to be not necessarily comparable with conspecifics - spawn mass numbers varied from one to eleven. HANOVA revealed between-individual variation in embryonic development time to be significant ( $P < 0.05$ , although it is notable that this was marginal). Comparison of the Mean Square and variance ratios shown in Table 2.3b indicates that a considerably greater proportion of the observed variation in embryonic developmental rate may be attributable to the between-population effect ( $P < 0.001$ ).

#### ii. Variation in Intracapsular Embryonic Period With Position in the Spawn Sequence

The maximum number of spawn masses laid within each population which developed to the hatching stage varied from a minimum of five produced by adults from Kinkell Braes, Robin Hood's Bay, and Loch Eriboll up to a maximum of eight laid by adults from Menai Bridge. Position in the spawn sequence was not found to exert a significant effect upon the duration of the embryonic development period ( $P > 0.10$ , Table 2.3c). The corresponding mean duration of the embryonic periods for sequentially laid spawn masses are presented in Table 2.3d. It is interesting that, whilst there is no significant trend, in four of the six populations (Robin Hood's Bay, Loch Eriboll, Menai Bridge and Portaferry) the shortest mean embryonic periods are apparent for the first-laid spawn masses, whilst the longest embryonic periods are evident in the later spawn masses (last, or second to last laid).

#### iii. Embryonic Period and Egg Size

Comparison of population mean egg volumes and population mean embryonic periods failed to indicate any trend reflecting the putative relationship between egg size and rate of embryonic development at the interpopulation level. The presence of any such relationship within each population was investigated by considering individual spawn mass embryonic period as a function of zygote diameter. The results of each simple linear regression are displayed in Table 2.3e, and graphically in Figures 2.4a-f. Only within the Cuan Ferry population was there found to be a significant linear relationship between egg size and duration of

**Table 2.3d. The Mean Duration of the Intracapsular Embryonic Periods of Sequentially Laid Spawn Masses from Kinkell Braes, Robin Hood's Bay, Loch Eriboll, Menai Bridge, Portaferry and Cuan Ferry.** The intracapsular embryonic period was defined as the number of days from spawning (uncleaved zygotes) to larval hatching. The mean values of sequentially laid egg masses are displayed. Integers in parentheses represent the number of spawn masses composing the appropriate mean.

Spawn Mass Sequence	Population					
	Kinkell Braes	Robin Hood's Bay	Loch Eriboll	Menai Bridge	Portaferry	Cuan Ferry
1 <sup>st</sup>	62.0 (3)	60.8 (8)	62.2 (12)	55.3 (3)	68.8 (8)	68.2 (13)
2 <sup>nd</sup>	60.2 (5)	63.9 (13)	64.4 (11)	64.2 (6)	69.0 (9)	72.2 (11)
3 <sup>rd</sup>	65.3 (4)	62.2 (9)	64.3 (8)	64.0 (7)	65.2 (9)	69.7 (7)
4 <sup>th</sup>	61.5 (2)	67.7 (3)	63.3 (3)	65.4 (7)	70.0 (2)	77.3 (6)
5 <sup>th</sup>	67.0 (1)	65.0 (3)	67.0 (1)	65.0 (2)	70.0 (1)	71.0 (2)
6 <sup>th</sup>	-	-	-	65.0 (3)	69.5 (2)	66.0 (1)
7 <sup>th</sup>	-	-	-	68.0 (2)	-	-
8 <sup>th</sup>	-	-	-	63.0 (1)	-	-

**Table 2.3c. Spawn Mass Embryonic Period as a Function of Mean Egg Diameter for Spawn Masses From Six U.K. Populations.** Embryonic Period is abbreviated to 'EP' and Egg Diameter to 'Egg Diam.'. The presence of a significant linear relationship is denoted by an asterisk.



Population	n	r Value	Regression Equation	<i>P</i> value
Cuan Ferry	14	-0.654	EP = -77 - 0.849 Egg Diam.	0.011*
Portaferry	8	-0.655	No significant correlation	0.078
Menai Bridge	11	-0.245	No significant correlation	0.467
Loch Eriboll	12	-0.173	No significant correlation	0.568
Robin Hood's Bay	13	-0.071	No significant correlation	0.828
Kinkell Braes	8	-0.307	No significant correlation	0.460

**Figures 2.4a-f. Regression of Spawn Mass Embryonic Period On Mean Spawn Mass Egg Diameter.** The results of regression analysis are displayed in Table 2.4b.

**Figure 2.4a. Cuan Ferry.** Cuan Ferry was the only population to exhibit a significant linear relationship (at the  $P < 0.05$  level) between spawn mass egg diameter and spawn mass embryonic period ( $n=14$ ).

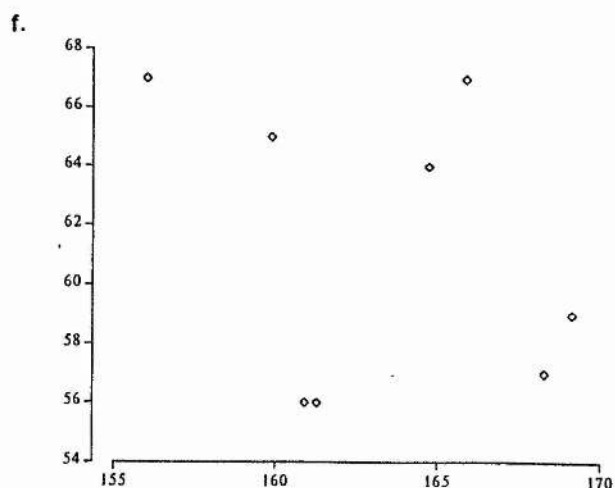
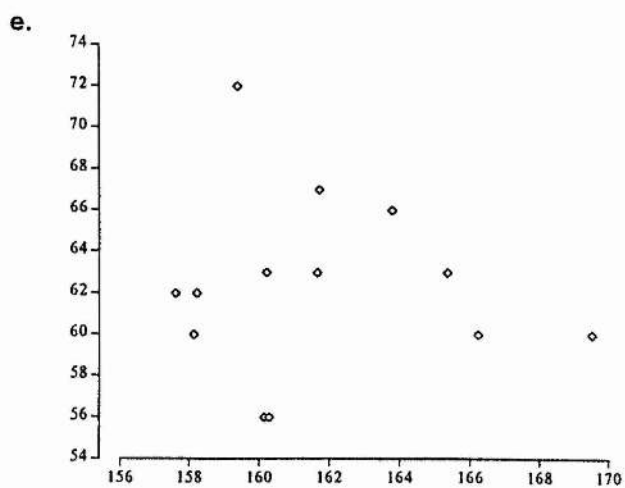
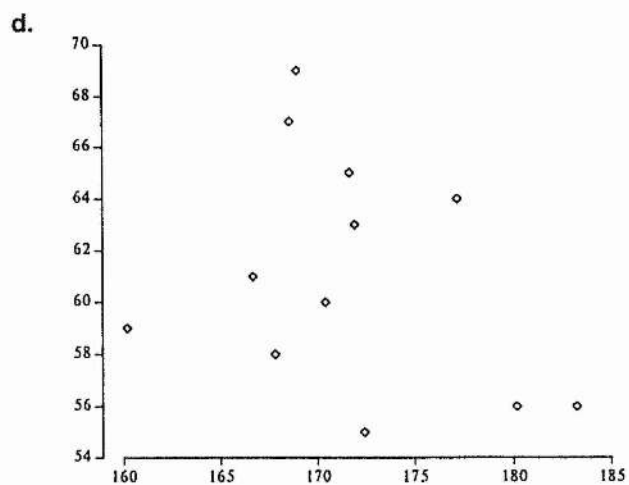
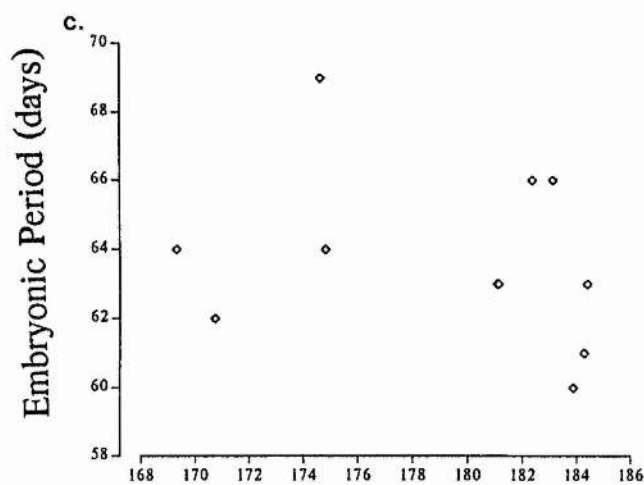
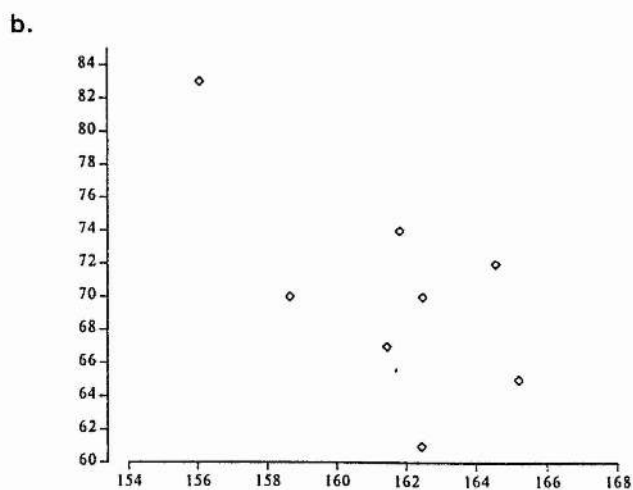
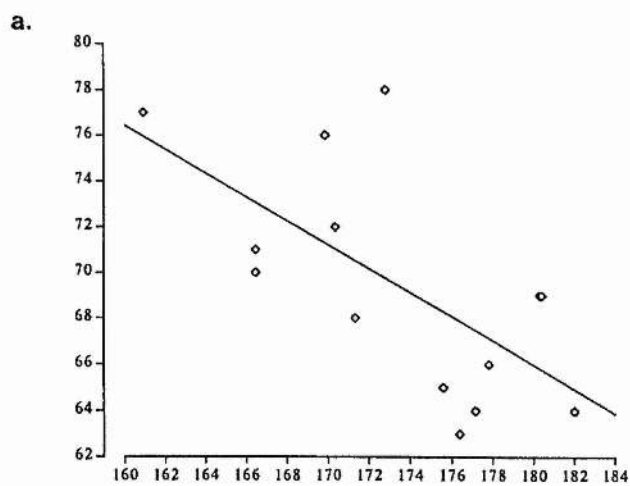
**Figure 2.4b. Portaferry.** ANOVA did not indicate a significant linear relationship between spawn mass egg size and spawn mass embryonic period to exist within this population ( $n=8$ ).

**Figure 2.4c. Menai Bridge.** ANOVA proved there to be no significant linear relationship between spawn mass egg size and spawn mass embryonic period in this population ( $n=11$ ).

**Figure 2.4d. Loch Eriboll.** ANOVA established that there was no significant linear relationship between spawn mass egg size and spawn mass embryonic period within this population ( $n=12$ ).

**Figure 2.4e. Robin Hood's Bay.** No significant linear relationship between spawn mass egg size and spawn mass embryonic period was evident within this population ( $n=13$ ).

**Figure 2.4f. Kinkell Braes.** ANOVA established there to be no significant linear relationship between spawn mass egg size and spawn mass embryonic period in this population ( $n=8$ ).



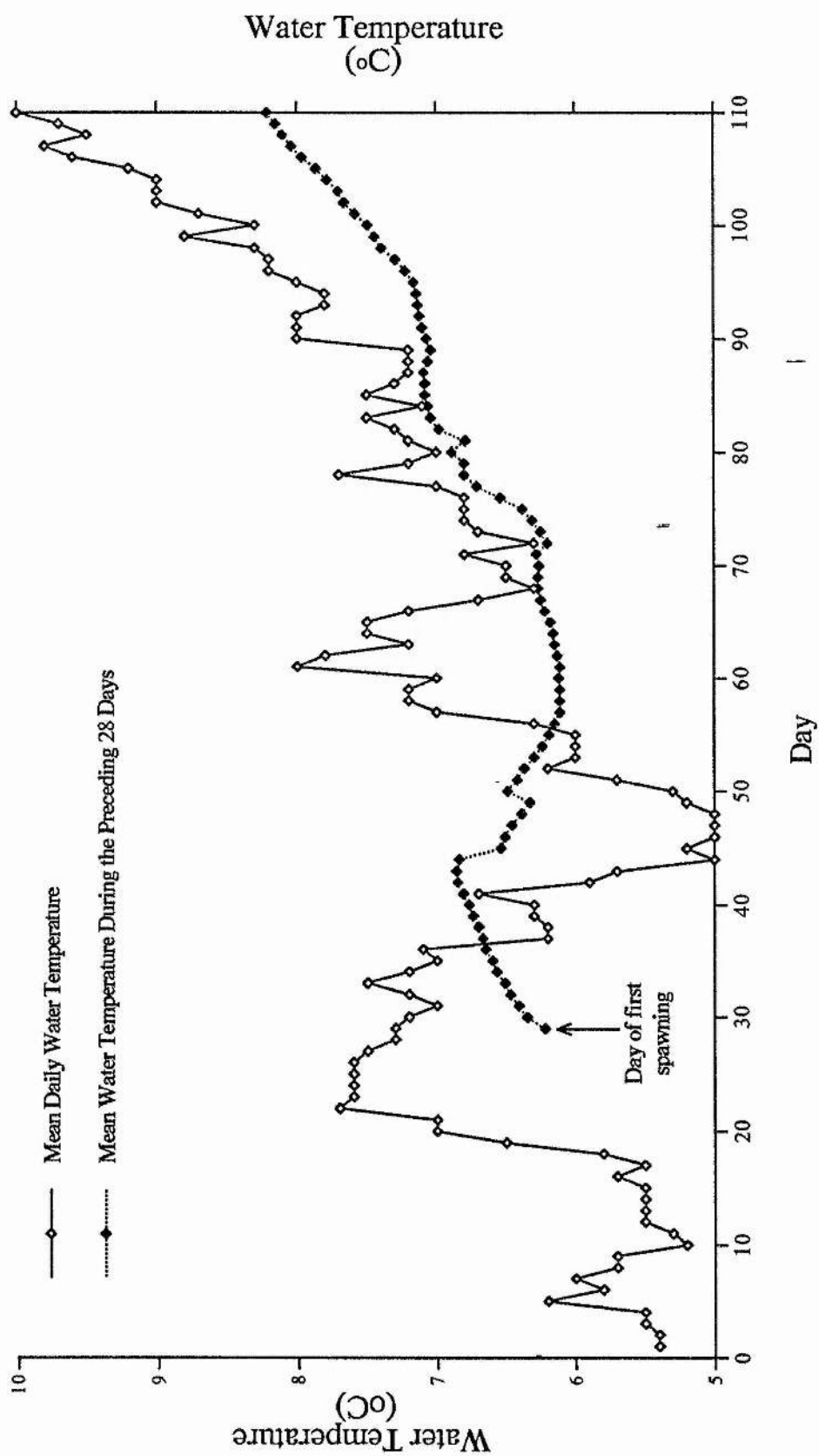
Zygote Diameter ( $\mu\text{m}$ )

the embryonic period ( $y = -77 - 0.849x$ ,  $r = -0.654$ ,  $P=0.011$ , see Table 2.3e). It is clearly evident from the extent of data scatter within Figures 2.4b-e, in addition to the low values of correlation coefficients and non-significant P-values (see Table 2.3e), that there is no such relationship between embryonic period and egg size within the remaining five populations.

#### iv. Water Temperature and Duration of the Intracapsular Embryonic Period

Whilst spawn masses were incubated at a constant temperature, parent adults were reared and maintained under ambient conditions within flowing seawater aquaria. The mean daily water temperature increased from 5.4°C to 10.0°C between 16<sup>th</sup> January (Day 1) and 5<sup>th</sup> May (Day 110) as shown in Figure 2.5a. It was therefore considered pertinent to assess whether the changing water temperature regime to which reproducing adults were subject had any effect upon the development of resulting progeny. The relationship between embryonic period and the mean water temperature to which parent adults were subjected in the 28d period prior to the production of the spawn mass was therefore assessed within each of the six *Adalaria proxima* populations maintained in the laboratory during the spring 1993 spawning season. Figure 2.6a shows a plot of the mean water temperature in the 28d period preceding production of each spawn mass, which can also be seen to increase from the first day of spawning (Day +28) until the last (Day 110). Before further analysis, each 28d block of daily temperature data from which the mean temperature in the preceding 28d had been calculated was subjected to, and passed, the Minitab correlation test for normality of distribution. Figures 2.5b-g show the duration of embryonic period plotted as a function of mean water temperature (in preceding 28d period) within each population. Regression analyses, shown in Table 2.3f established a significant positive linear relationship to exist between the duration of the embryonic period and mean water temperature (in the preceding 28d period) within four of the six populations studied (Portaferry and Menai Bridge constituting the exceptions). Analysis of covariance (ANCOVA) by the general linear model (GLM) method indicated that water temperature (in the 28 d preceding spawning) did not differ in its effect between populations ( $F=0.34$ ,  $P=0.799$ ). The water temperature to which the adult is subjected in the 28 days prior to spawning does therefore appear to exert a uniform effect upon embryonic developmental rate of progeny, at least within four of the six populations studied (Cuan Ferry, Loch Eriboll, Robin Hood's Bay and Kinkell Braes).

**Figure 2.5a. The Mean Daily Water Temperature at Which Adult *Adalaria proxima* From 6 U.K. Populations Were Maintained Over the Course of the Spawning Season.** Daily temperatures cited are the means of three daily observations. The daily water temperature data displayed is numbered sequentially beginning at Day 1 on 16<sup>th</sup> January (28 days prior to the laying of the first spawn mass on 13<sup>th</sup> February ) and ending at Day 110 on 5<sup>th</sup> May , when the last spawn mass was produced. In addition, the mean water temperature to which adults were subjected in the 28d period immediately prior to spawning is shown from 13 February, when spawning commenced, until 5<sup>th</sup> May, when spawning finished.



**Figures 2.6b-g. The Relationship Between the Duration of the Embryonic Period and Mean Water Temperature in the 28 day Period Preceding Spawning.** Adult *Adalaria proxima* were maintained within the same tanks and were therefore subject to similar conditions of fluctuating water temperature over the spawning season. Both the daily water temperature over the spawning season and the mean temperature in the 28 days preceding spawning are illustrated in Figure 2.5a. The results of regression analysis are displayed in Table 2.3f.

**Figure 2.5b Cuan Ferry.**

**Figure 2.5c Portaferry.**

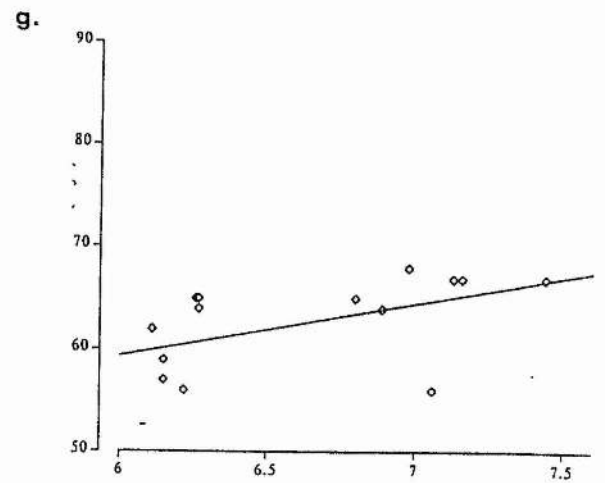
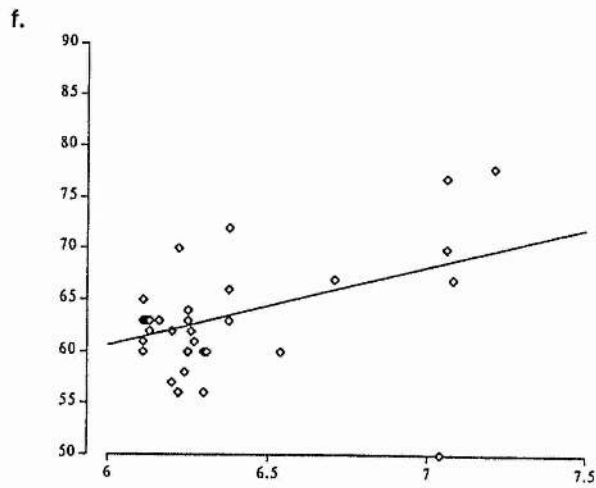
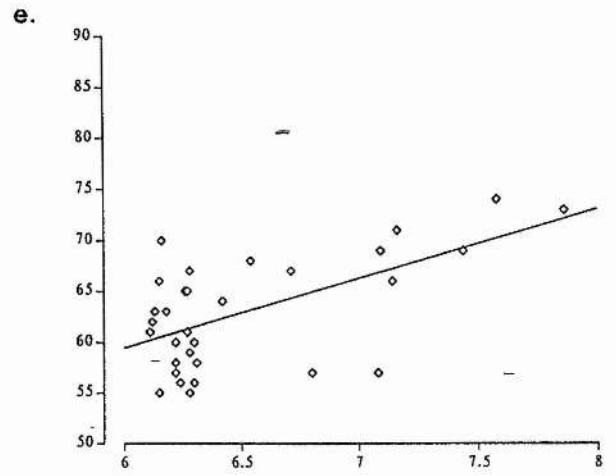
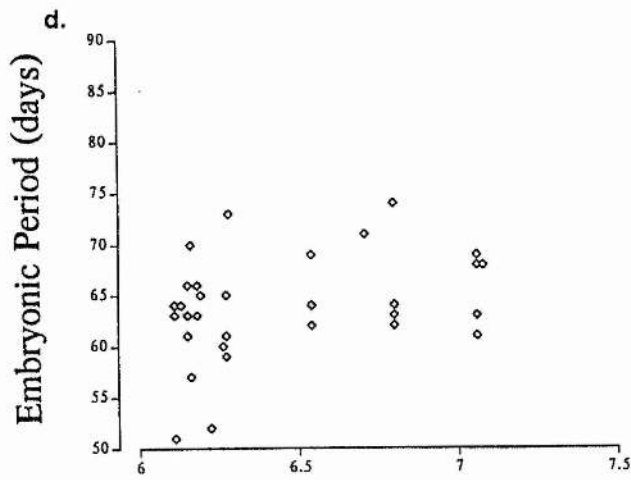
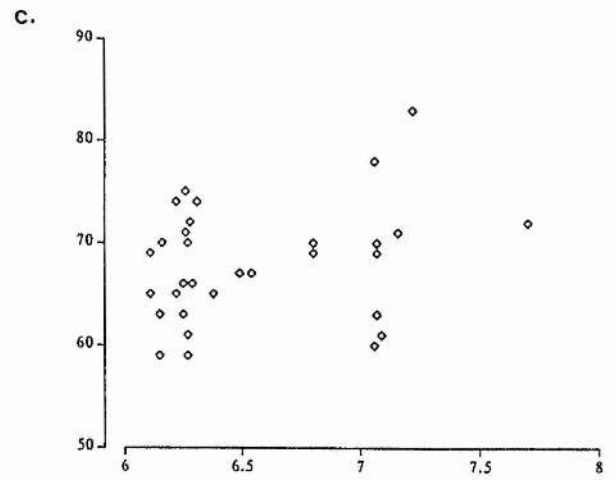
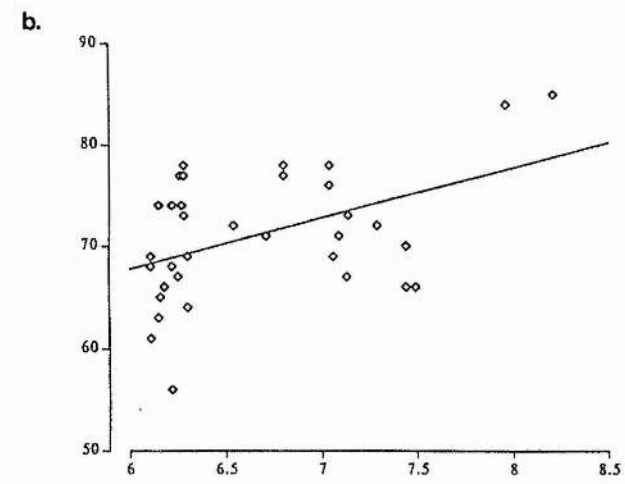
**Figure 2.5d Menai Bridge**

**Figure 2.5e Loch Eriboll**

**Figure 2.5f Robin Hood's Bay.**

**Figure 2.5g Kinkell Braes.**





Water Temperature in 28d Preceding Production of Spawn Mass ( $^{\circ}\text{C}$ )

**Table 2.3f. Regression Equations for Embryonic Period as a Function of Water Temperature for all Six U. K. Populations of *Adalaria proxima*.** 'Water temperature' represents the mean water temperature (°C) to which adults were subjected during the 28 days prior to production of the relevant spawn mass. A significant linear relationship between length of the embryonic period and water temperature within a population is denoted by an asterisk. Both the daily water temperature and the mean water temperature to which adults were subjected during the 28 d before spawning are displayed in Figure 2.5a.

Population	r Value	Regression Equation	<i>P</i> value
Cuan Ferry	0.427	EP = 37.8 + 5.00 T	0.004*
Portaferry	0.200	No significant correlation	0.145
Menai Bridge	0.288	No significant correlation	0.064
Loch Eriboll	0.593	EP = 18.6 + 6.82 T	<0.001*
Robin Hood's Bay	0.395	EP = 16.8 + 7.31 T	0.010*
Kinkell Braes	0.483	EP = 28.3 + 5.18 T	0.039*

### Variation in Hatching Success at the Intra- and Interpopulation Levels

The hatching success (expressed here as back-transformed percentages) of a total of 294 spawn masses from six populations of *Adalaria proxima* were determined during the course of this study. Hatching success was defined as the proportion (in percentage terms) of embryos which successfully emerged from the spawn mass as veliger larvae. Observed levels of hatching success ranged from total spawn mass hatching failure to 100% hatching success. The population means were comprised of a minimum of 19 spawn masses (Menai Bridge) up to a maximum of 89 spawn masses (Cuan Ferry), and back-transformed mean population levels of hatching success (displayed in Table 2.4a) varied from 11% in spawn masses from Portaferry up to 62% in spawn masses from Kinkell Braes. Further, variance in mean hatching success was observed to be high within all populations (see Table 2.4a).

The disparate mean levels of population hatching success were further addressed by considering the possible role of the individual and of position in the spawn sequence as causative factors. Inclusion in the analysis of within-individual variation in hatching success was precluded by inadequate replication - the hatching success of each spawn mass was determined as a whole, and therefore denoted by just one integer. A significant proportion of the observed variation in hatching success was found to be attributable to population ( $P < 0.025$ , see Table 2.4b). In contrast, the variation shown between individuals nested within populations was established to be nonsignificant ( $P > 0.25$ , see Table 2.4b also). The minimum spawn mass sequence for which hatching success was determined was five in the Kinkell Braes population, whilst the maximum was provided by the Cuan Ferry population which laid a total sequence up to 11 spawn masses. The back-transformed mean levels of hatching success shown by six populations of *Adalaria proxima* are shown in Table 2.4d, and there appears a clear visible trend of rapidly decreasing hatching success with increasing spawn mass number. Table 2.3c shows that spawn mass hatching success did indeed vary significantly ( $P < 0.025$ ) with position in the spawn sequence. Within all populations, the maximum mean levels of hatching success were displayed by the first and second-laid spawn masses, the highest level (93.5%) being attributable to the Kinkell Braes population. As the spawning season progressed adults died,

**Table 2.4a (top) Mean Levels of Hatching Success For Six U.K. Populations of *Adalaria proxima*.** Populations are ranked in descending order of hatching success. Hatching success was defined as the proportion (in percentage terms) of embryos to successfully hatch as veligers from spawn masses cultured at 6°C. These values were arc-sine transformed before statistical analysis and the resulting back-transformed population means are presented within this table. The column titled 's.e.' denotes the appropriate values of standard error. The number of spawn masses composing the appropriate mean is indicated by the column headed 'n'.

**Table 2.4b (middle). Hierarchical ANOVA (HANOVA) Examining The Effect of Population And Individual On Hatching Success.** Asterisks denote results considered to be significant (at the  $P < 0.05$  level).

**Table 2.4c (top). HANOVA Examining The Effect of Population And Spawn Sequence On Hatching Success.** Asterisks denote results considered to be significant .

Population	Mean Hatching Success		
	n	(%)	s.e.
Kinkell Braes	20	62.4	49.3-74.6
Loch Eriboll	48	43.4	33.6-53.5
Robin Hood's Bay	42	34.7	25.5-44.6
Menai Bridge	19	33.1	19.8-47.7
Cuan Ferry	89	16.8	12.4-21.8
Portaferry	76	11.1	7.4-25.0

	df	SS	MS	Variance ratio	P
<i>Source</i>					
Between Population	5	29293	5859	4.13	<0.025*
Between Individual	75	106515	1420	1.19	>0.25
Within Individual	212	253220	1194	-	-
Total	292	389028	1332	-	-

	df	SS	MS	Variance ratio	P
<i>Source</i>					
Between Population	5	29293	5859	2.93	<0.025*
Between Spawn Sequence	38	75863	1996	1.75	<0.025*
Within Spawn Sequence	249	283872	1140	-	-
Total	292	389024	1332	-	-

**Table 2.4d. The Mean Hatching Success of Spawn Masses originating from Kinkell Braes, Robin Hood's Bay, Loch Eriboll, Menai Bridge, Portaferry and Cuan Ferry as a Function of Position in the Spawn Mass Laying Sequence.** Adult *Adalaria proxima* did not necessarily produce similar numbers of spawn masses when compared to peers both within and between populations. Individuals laid between one and eleven spawn masses over the course of the reproductive season. The back-transformed mean levels of hatching success for sequentially laid spawn masses within each population are displayed here. Integers in parentheses represent the number of spawn masses composing the appropriate mean.



Back-transformed Mean Hatching Success  
(%)

Spawn Mass Sequence	Kinkell Braes	Loch Eriboll	Robin Hood's Bay	Menai Bridge	Cuan Ferry	Portaferry
1 <sup>st</sup>	47.6 (5)	54.7 (14)	45.1 (9)	30.8 (4)	37.4 (15)	68.6 (9)
2 <sup>nd</sup>	93.5 (6)	40.1 (10)	63.6 (14)	31.0 (4)	59.5 (15)	31.3 (14)
3 <sup>rd</sup>	32.6 (5)	47.9 (10)	24.0 (13)	30.3 (5)	19.4 (13)	22.9 (11)
4 <sup>th</sup>	81.6 (2)	38.6 (4)	5.0 (7)	60.6 (3)	18.8 (13)	1.0 (12)
5 <sup>th</sup>	34.1 (2)	18.8 (3)	34.2 (4)	0 (1)	3.5 (12)	1.2 (12)
6 <sup>th</sup>	-	0 (1)	0 (1)	38.8 (2)	0.3 (8)	0.7 (8)
7 <sup>th</sup>	-	-	-	-	0 (6)	0 (4)
8 <sup>th</sup>	-	-	-	-	0 (3)	0 (3)
9 <sup>th</sup>	-	-	-	-	0 (2)	0 (2)
10 <sup>th</sup>	-	-	-	-	0 (1)	-
11 <sup>th</sup>	-	-	-	-	0 (1)	0 (1)

resulting in progressively lower sample sizes. Whilst interpretation of data must therefore become appropriately more conservative, it is notable from Table 2.4d that spawn masses laid sixth or later in the spawn sequence rarely hatched successfully.

The finding that hatching success decreases with increasing spawn mass number may be important when comparing the overall mean hatching success of populations. It is clear from this study that the number of spawn masses laid by individual adults may differ considerably. In a population, such as that of Portaferry, in which more spawn masses are laid per adult, the overall mean hatching success may be artificially depressed by the reduced hatching success characteristic of later spawn masses. A proportional measure of hatching success, whilst a useful indicator when considered in concert with other reproductive parameters, may therefore be too crude a tool to compare the relative production of progeny between populations, lacking as it does any indication of absolute numbers.

#### Interpopulation Variation in Larval Shell Length

The larval shell lengths of progeny from five populations of *Adalaria proxima* were determined (Table 2.5a). Mean veliger shell length ranged from a minimum mean of 243µm in larvae originating from Portaferry, up to a mean of 270µm in larvae originating from Cuan Ferry. ANOVA established there to be a significant difference between population mean shell lengths ( $F_{4,515}=36.0$ ,  $P<0.001$ ). Population mean shell lengths are illustrated, with appropriate Tukey groupings, in Figure 2.6a. The largest shell sizes were exhibited by larvae from both Cuan Ferry (270µm) and Loch Eriboll (266µm). Larval shells from the Robin Hood's Bay population, at 259µm, were significantly smaller than those from Cuan Ferry, although not judged to be significantly smaller than those of the Loch Eriboll population. The minimum shell lengths, significantly smaller than those exhibited by all other populations, were associated with Kinkell Braes (245.2µm), and Portaferry (243µm).

A general trend of decreasing population mean larval shell length with decreasing population mean egg volume is apparent within this study. With the exception of Kinkell Braes (for which the mean population shell length was

**Table 2.5a (above) The Larval Shell Length of Progeny From Five U.K. Populations of *Adalaria proxima*.** Populations are shown in descending magnitude of larval shell length. Data are the mean values derived from the measurement of 20 larval shells per individual spawn mass. Population mean larval shell lengths are displayed at the foot of the table. Values in parentheses denote the number of spawn masses composing the population mean.

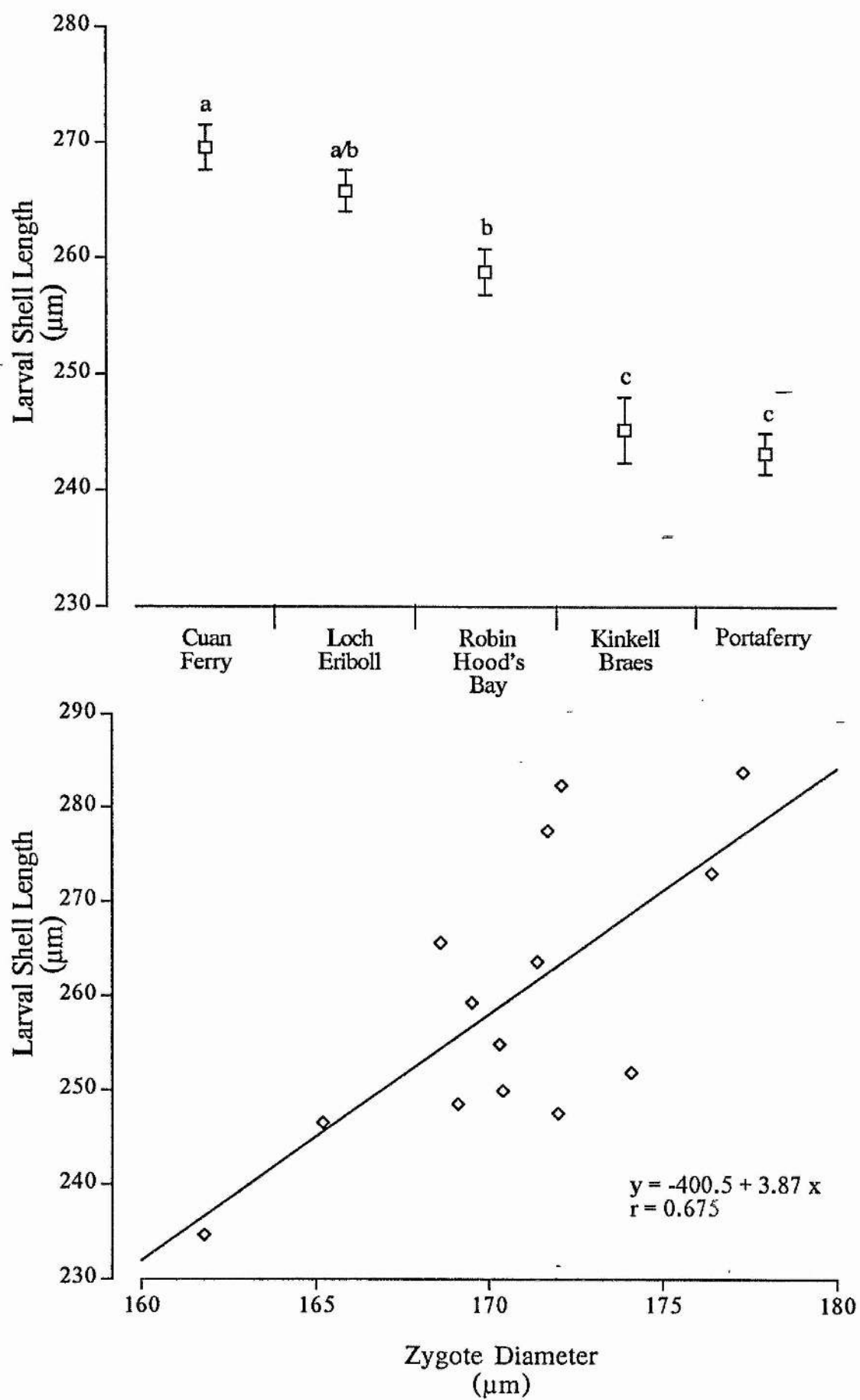
**Table 2.5b (below). ANOVA Table of Mean Larval Shell Lengths between five U.K. *Adalaria proxima* populations.** A significant difference in mean larval shell length between populations is evident ( $P < 0.001$ ). The relevant absolute values of mean larval shell lengths are displayed in Table 2.5a.

Spawn Mass	Larval Shell Length ( $\mu\text{m}$ )				
	Cuan Ferry, Argyll	Loch Eriboll, Sutherland	Robin Hood's Bay, N. England	Kinkell Braes, Fife	Portaferry, N. Ireland
1	259	262	259	246	256
2	283	277	264	248	234
3	254	265	246	240	246
4	273	247	265	-	230
5	282	250	-	-	252
6	263	278	-	-	238
7	-	278	-	-	-
Mean	269.5 (6)	265.7 (7)	258.8 (4)	245.2 (3)	243.2 (6)

	df	SS	MS	F-value	P
between populations	4	60607	15152	36.0	<0.001*
within populations	515	216772	421		

**Figure 2.6a. The Mean Larval Shell Length of Five United Kingdom Populations of *Adalaria proxima*.** The corresponding absolute values and sample sizes are presented in Table 2.5a. One-way ANOVA established there to be a significant difference in larval shell length between populations (see Table 2.5b.) Superscript type denotes groupings resulting from *a posteriori* multiple comparison by Tukey's HSD. Groupings are considered significantly different at the  $P < 0.05$  significance level.

**Figure 2.76. Regression of Larval Shell Length as a Factor of Zygote Diameter.** The total number of individual spawn masses for which both larval shell length and mean zygote diameter were obtained was 14. Spawn mass zygote diameters are mean values derived from 55 zygotes measured at random within a spawn mass. Larval shell length values represent the mean shell length of 20 larvae from the relevant spawn mass. Regression ANOVA established there to be a significant linear relationship between mean spawn mass zygote diameter and the shell length of resulting progeny ( $F_{1,12} = 11.84$ ,  $P < 0.001$ ). The Model II regression equation and appropriate correlation coefficient are displayed.



derived from the minimum number of just three spawn masses) each population's ranking in egg volume matches its ranking in shell size. The putative relationship between zygote diameter and egg volume was investigated in a more refined manner by performing Model II Regression analysis upon the zygote diameter and shell length of resultant larvae for individual spawn masses (independent of population). A significant positive linear relationship ( $y = -400.5 + 3.87x$ ,  $r = 0.675$ ) between zygote diameter and larval shell length was established, and is illustrated in Figure 2.6b.



## 2.4 DISCUSSION

### Interpopulation Variation in Egg Size and Number

Significant variation both in spawn mass egg size and number was found to occur at the interpopulational level in *Adalaria proxima* originating from eight sites within this species' distribution range around the northern coasts of Britain. Adults from Loch Eriboll, Sutherland, produced the largest spawn masses overall, containing a mean of 1792 eggs, whilst adults from Portaferry, N.Ireland, laid the smallest containing a mean of just 810 eggs. Spawn masses produced by adults from Portaferry, contained the smallest eggs, with an overall mean volume of just  $2.15 \cdot 10^{-3} \text{mm}^3$  (that of first-laid spawn masses being  $2.269 \cdot 10^{-3} \text{mm}^3$ ), whilst spawn masses from Menai Bridge, Anglesey were found to contain the largest eggs at  $3.10 \cdot 10^{-3} \text{mm}^3$  (first-laid mean  $3.276 \cdot 10^{-3} \text{mm}^3$ ). No trend between the mean egg size and number of eggs per spawn mass was evident between populations within the present study. Egg 'size' has been widely expressed both in terms of ovum or zygote diameter and egg volume within the literature. Many authors, when considering the effects and significance of variation in egg size have preferentially employed egg volume, considering it a more functional indicator of size than is egg diameter (Strathmann & Vedder, 1977; Kaplan, 1980; McEdward & Coulter 1987; Clarke & Gore, 1992; Baur, 1994). In addition, the use of egg volume allows comparison between spherical, ovoidal and spheroidal forms of eggs (Turner & Lawrence, 1979). Egg size is accordingly considered as volume throughout the present study. It is important to consider the considerable differences in egg volume conferred by apparently minimal differences in egg diameter. For example, whilst the difference in diameter between the smallest and largest overall mean population egg diameters is a mere  $20.8 \mu\text{m}$ , the size of Portaferry eggs constitutes only 69% of that of Menai Bridge eggs when compared in volumetric terms.

The significant differences in mean population egg sizes and numbers may not be attributable to disparate adult sizes between populations. In a comparative study of reproduction by *Adalaria proxima* and the sympatric dorid *Onchidoris muricata*, Todd (1987) found the reproductive output of adults to

vary in an unpredictable manner independent of adult body size. In addition, Havenhand & Todd (1988b) also observed that total egg production does not simply correlate with maximum body size in *Adalaria proxima* and concluded fecundity to be affected by factors other than the mass of the egg producing adult.

Intraspecific latitudinal clines in egg size have been established within the Class Crustacea, within the Isopoda (Clarke & Gore, 1992); Cirripedia (Patel & Crisp, 1960) and most extensively, the Decapoda (Clarke, 1979; Clarke *et al.*, 1991; Gorny *et al.*, 1992). For example, populations of the deep water prawn *Pandalus borealis* have been reported to show both increased egg volume and egg organic content with increasing latitude, irrespective of adult size (Clarke *et al.*, 1991).

Within the present study selected experimental populations of *Adalaria proxima* were sited around the northern British coast covering 5° 16' latitude from Menai Bridge to Loch Eriboll and 4° 40' longitude from Portaferry to Robin Hood's Bay. It is apparent that populations from the North Sea coast of the U.K. showed similar egg sizes. On the west coast of Britain populations also possessed egg sizes which were not significantly different to each other, and which tended to be larger than those of the east coast populations. The Portaferry population appears distinct from the others in two ways. First, this site is the only one not situated on the British mainland, and is therefore geographically isolated from the others by the Irish Sea. Second, this site is characterised by eggs which are significantly smaller than those of all the west coast populations (Menai Bridge, Cuan Ferry, Clachan Seil, and including Loch Eriboll), but are similar in size to those of the more distant populations on the east coast of the U.K. (Robin Hood's Bay, Kingsbarns and Kinkell Braes). However, despite these tentative groupings, no latitudinal/clinal relationship in mean egg volume between populations was evident between the sites surveyed.

Variation in egg size between populations of conspecifics has also been attributed to food supply and quality. For example, the fresh water shrimp *Palaemon paucidens* exhibits considerable differences in egg size between populations found in Japanese inland waters (Nishino, 1980). In a survey of 27 populations Nishino (1980) found egg size to differ by as much as a factor of seven between populations yet remain consistent, irrespective of adult body size, within populations. A later study focusing on populations inhabiting distinct

habitats within the Sagami River found that mean egg size was significantly larger in populations inhabiting the lower, faster flowing, waters of the river than those inhabiting the standing waters nearer the source of the river. This inter-population variation in egg size was interpreted by the author as an adaptive response to larval food availability. In the upper river a greater amount of organic particulates were proposed to be available than in the lower region, favouring the production of more numerous, smaller larvae and thus smaller eggs.

Interpopulation egg sizes due to adaptation to differential larval food availability has also been reported among marine invertebrates. George *et al.* (1990) found that the echinoid *Arbacia lixula* increases egg quality (size and organic content) under favourable conditions. The eggs, which give rise to planktonic larvae, were consistently smaller, in addition to possessing higher lipid and protein contents, at Cap Ferrat (France) when compared to those at Villefranche (France). The authors postulated this to constitute a response to the higher proportion of the preferred algal food source present at Cap Ferret.

Selection favouring differential interpopulation egg sizes has also been attributed to abiotic environmental conditions. In a study of two northern and southern United Kingdom populations of the amphipod *Gammarus duebeni*, Dunn & McCabe (1995) demonstrated that the northern population at Budle Bay, Northumberland, laid significantly larger eggs than the population from Totton Marsh, Hampshire. This inter-population difference in egg size was interpreted to be an adaptive response to the contrasting environments to which each population was subject. However, only two survey sites were included within the study, and any observed or implied trend should therefore perhaps be regarded with caution. Such is the case for the asteroid echinoderm *Leptasterias epichlora* (George, 1994). Females from an exposed site were observed, both in the field and in the laboratory, to produce larger (and more) eggs containing a greater quantity of protein per egg than did females from a site considered to be sheltered. The production of larger and more eggs in individuals subject to favourable environmental conditions, shown by George *et al.* (1991) and George (1994) do not concur with the predictions of many standard life-history models (Vance, 1973a; Smith & Fretwell, 1974; Brockleman, 1975; Wilbur, 1977; see review of Roff, 1992). Such life history models incorporate the central assumption that the energy available for reproduction is finite, and therefore an increase in egg size (and hence *per capita* energetic investment) must

result in a decrease in egg numbers. These models address the evolution of interspecific allocation of reproductive resources to egg size and number. The studies of George *et al.* (1991) and George (1994) may thus be regarded as providing an example of the invalidity of applying such models when considering phenotypic plasticity between geographically separated populations of conspecifics.

Egg number is not a conservative parameter for a species and may vary with factors such as individual adult nutritional state and population density (Turner & Lawrence, 1979). Within the present study, those populations which possessed the largest mean egg size did not necessarily produce fewer eggs, as would be predicted by the models of Vance (1973a), Smith & Fretwell (1974) and Brockleman (1975). However, neither are the observed relationships between population mean egg size and number, with the exception of the Portaferry population, consistent with the findings of George *et al.* (1991) and George (1994). For example, although adults associated with Portaferry produced spawn masses containing the smallest eggs, spawn masses from both the Cuan Ferry and Menai Bridge populations contained a statistically similar number of eggs to Portaferry, yet the eggs were significantly larger. This would intimate that at the interpopulation level there may be additional and possibly different factors governing the optimum spawn mass egg size and number within each population of *Adalaria proxima*. For example, the environmental conditions to which reproducing adults and resulting larvae are exposed will not be uniform across sites. In addition, the optimal egg size and number may be affected by population density and larval mortality, which again may be distinct between sites (Parker & Begon, 1986; McGinley, 1989; Sargent *et al.*, 1987). Larvae resulting from smaller eggs, depending upon environmental conditions, may have the same level of fitness as those from larger eggs (Sinervo, 1990; George, 1994). For example, Portaferry larvae originating from smaller eggs may, if offspring competition is low, have an equivalent level of fitness to that of offspring from larger eggs subjected to a site where competition is higher. This does assume however that small larvae would not be out-competed by larger chance migrants which would subsequently alter the population gene pool.

### Intrapopulation Variation in Egg Size and Number

Egg volume was not uniform within populations and considerable interspawn mass variation was observed. *Adalaria proxima* adults may lay several (up to 11 in the laboratory) spawn masses over the course of the reproductive period. The rôle of both the individual and spawn mass position in the laying sequence was therefore considered as a possible determinant factor acting upon egg size. The effect of individual upon egg size was found to be non-significant ( $P=0.130$ ). However, spawn mass sequence was established to exert a significant effect upon egg size ( $P<0.001$ ). For example, within four of the six populations studied maximum egg volumes were evident among the first-laid spawn masses whilst the minimum egg volumes were consistently observed to occur towards the end of the spawning sequence. Further, egg size decreased with increasing position in the spawn sequence in a uniform manner across all populations. These results concur with the subjective findings of Thompson (1958) who observed that mean egg diameter tended to decrease throughout the spawning season.

Parker & Begon (1986) constructed a model which examined the optimal clutch and egg size for sequentially reproducing insects under variable environmental conditions. Such a theoretical model concerning intraspecific variation in egg size over a single spawning season is unusual within the literature. Whilst formulated with reference to insects with separate feeding and breeding grounds, it may be instructive when applied to marine invertebrate egg size. The authors predicted both egg and clutch size to remain constant with increasing number of clutches. However the findings of the present study indicate that *Adalaria proxima* does not conform to these predictions and thus may be subject to a different hierarchy of selective pressures than those considered in the model of Parker & Begon (1986).

There are numerous examples within the literature of a variation in egg size amongst marine invertebrates over the course of the spawning season, and both adaptive and non-adaptive explanations have been proposed for the phenomenon. For example, the polychaete *Capitella* sp. produces pelagic lecithotrophic larvae and reproduces on average three times (exceptionally five times) before death. In a study by Qian & Chia (1992) egg size was demonstrated to decrease significantly after the first spawning, and egg energetic content to consistently decrease after the second. The authors concluded this



decrease in *per capita* energetic content may have been attributable to the gradual depletion of the energy available to reproduction. Alternatively Qian & Chia (1992) proposed the decrease in egg size and energetic content to increase fecundity which would confer, if reduced egg size had no negative effects on subsequent life history parameters, increased reproductive success. Whether this decrease in egg size and energetic content over sequential spawning constituted an adaptive response or was non-adaptive could not be determined in the absence of larval and juvenile fitness data. In a similar manner the egg size of the butterflies *Pararge aegeria*, *Lasiommata megera* and *Epirrita autumnata* have been documented to decrease over the course of the laying season (Karlsson & Wiklund, 1984; Ruohomäki *et al.* 1993). A significant change in egg size may not always be adaptive however, and indeed, in this case Karlsson & Wiklund (1984) proposed the decrease in egg size to be more probably merely a function of parent inability to partition reproductive resources accurately. That an intraspecific egg size variation may not necessarily be of adaptive significance, and may rather be due to the adult being physiologically incapable of regulating egg size has also been proffered in explanation for observed intraspecific variation in several marine invertebrate species (McEdward & Carson 1987, McGinley *et al.* 1987).

Seasonal variability of egg sizes within successive spawn masses may be selected for if the adult was subjected to a temporally varying environment and had the means to both assess or predict conditions and consequently adjust optimum egg size (McGinley *et al.* 1987). This could only be considered of advantage however if conditions to be encountered by the parent were an accurate predictor of the environment encountered by the larvae (Brody & Lawlor 1984). An adaptive response to varying predation patterns has been attributed to both the decline in egg size through season observed in the deep water prawn *P. borealis* (Clarke *et al.* 1991) and in a cladoceran (Kerfoot 1974). The direct developing isopod *Armillidium vulgare* exhibits a decline in egg diameter through spring to summer, a trend attributed to the concomitant increase in food resources available to the larvae (Brody & Laylor 1984). Kaplan & Cooper (1984) considered that seasonal environmental variation selected for the production of variable egg sizes; however, McGinley *et al.* (1987) attributed this instead to weaker selection and proposed it to be the within year variation in offspring size which shows an adaptive response.

All *Adalaria proxima* populations within the present study showed a significant decrease in the number of eggs contained within each spawn mass as position in the laying order increased. The results were similar to those concerning egg size: namely that whilst a significant proportion of observed variation in egg number was attributable to the effect of position in the spawn sequence ( $P < 0.001$ ) variation at the between-individual level was considered to be non-significant ( $P > 0.25$ ). The observed decrease in egg number between the first and last-laid spawn mass was relatively consistent between populations, the greatest decrease of 86.4% being shown by the Portaferry population whilst the smallest decrease of 70.4% was attributable the Robin Hood's Bay population. Again, within all populations the greatest reproductive investment was directed into the first-laid spawn masses, which constituted between 46% (Kinkell Braes) and 26% (Portaferry) of total mean egg production within each population. These results are consistent with the observations of Thompson (1967) who reported that the largest egg masses appeared to be produced by *Adalaria proxima* at the start of the spawning season, although no quantitative measures were presented. In addition, using dry weight conversions of spawn mass, Havenhand & Todd (1988b) also reported the larger proportion of total spawn production (31.7%) to be invested within the first spawn mass.

The additional energy required to produce an egg decreases as overall spawn mass egg number increases. Havenhand & Todd (1988b) proposed that the reproductive strategy of *Adalaria proxima* has evolved such that, after production of the first spawn mass, the number of eggs contained within each spawn mass will sharply decline to the minimum possible without compromising the energetic efficiency of egg production. Todd (1987) examined the energetics of egg production in *A. proxima* and proposed there to be a threshold number (630) of eggs within a spawn mass at which the optimal 'cost per egg' to be reached. Below this threshold number the cost of 'packaging' each egg in the protective gelatinous spawn mass stroma resulted in egg production becoming increasingly inefficient. Whilst this was consistent with the mean number of eggs per spawn mass (675) observed within the study of Todd & Havenhand (1988b), it is not consistent with the present study. The mean number of eggs observed within spawn masses was considerably greater and varied between populations from 1792 (Loch Eriboll) down to a minimum of 810 (Portaferry). In addition, after production of the first spawn mass, the number of eggs in subsequent spawn masses was not observed to drop sharply to the plateau level of around 630 eggs predicted by Havenhand &



Todd (1988b) within any of the six populations studied. In contrast, there appeared a trend of gradually decreasing egg number with increasing position in the laying sequence, with only fourth or fifth-laid spawn masses containing the predicted minimal optimal egg number of between 600-700.

The observed decrease in both egg size and egg number in *Adalaria proxima* with increasing spawn sequence may be attributable to several causes. For example, it may be that the significant decrease in both the egg size and number of progeny within spawn masses over the course of the reproductive season constitutes an adaptive response by adults to changing environmental conditions. It is notable however that the reduction in fecundity and egg size is evident within all populations. The unified nature of this trend of decreasing egg volume and number across populations may be indicative of a gradual depletion in available reproductive resources over the course of the reproductive season. Certainly, the significant decrease observed in the number of eggs contained within spawn masses would offer support for this nonadaptive explanation, as would the subjective judgement of egg quality in *A. proxima* reported by Thompson (1958). He observed a gradual shift in ova colour from 'yellow to cream' in spawn produced early in the season, tending towards a paler cream later. This was interpreted to indicate a decrease in quality/quantity of yolk over the spawning period, which would be consistent with the evidence of the present study in indicating the effects of a depletion in energetic reserves available for reproduction. Qian & Chia (1992) proposed the decrease in egg size over the spawning season evident in the polychaete *Capitella* sp. could be attributed to the depletion of parental energy available for reproduction. Alternatively, Turner & Lawrence (1979) postulated egg numbers may be decreased in order to retain egg quality. It would appear that *A. proxima* employs a combination of these strategies in order to compensate for the depletion of energetic reserves later in the season. Additional evidence that *A. proxima* cannot sustain the energetic requirements of repeated spawning is provided by the work of both Thompson (1958) and Havenhand & Todd (1988b). After production of the last spawn masses *A. proxima* rapidly degenerate and die (Thompson, 1958; Todd, 1987). However, progressive degeneration of body tissue in reproducing adults was observed by Thompson (1958) over the course of the spawning season. This 'degrowth' was confirmed by Havenhand & Todd (1988b) who considered it to represent the catabolism of functional proteins. Such degrowth among nudibranchs has been proposed as a possible source of energy for reproduction (Clark, 1975). Thus, Havenhand & Todd (1988b) concluded

degrowth in *A. proxima* to supplement the reproductive energetic reserves depleted by repeated spawning.

#### The Ecological Significance of Variation in *Per Capita* Investment

The significance of a variation in egg energetic content will not be uniform across classes, or even between species. Variation in propagule size may, for example, be an adaptive response to predictable fluctuating environmental conditions affecting larval survival (Capinera, 1979; Brody & Lawlor, 1984; Kolding & Fenchel, 1981, Dunn & McCabe, 1995). The influence of a changing environment was not considered in the model of Smith & Fretwell (1974) which favoured a constant *per capita* investment in progeny. McGinley *et al.* (1987) constructed a model encompassing temporally and spatially varying environments to examine under which conditions intra-clutch variation in egg size would be selected for. It assumed that offspring in a 'good' environment would need less parental investment to successfully recruit relative to those in a 'less good' environment. McGinley *et al.* (1987) accordingly proposed that in a spatially varying environment with high density dependent loss of offspring, variation could be selected for if dispersal was non-random.

For these egg size measurements to be of any ecological significance in *Adalaria proxima* egg diameter must be a indicator of egg organic content, and thus parental energetic investment. Egg size is widely accepted to be a reliable reflection of organic content at the interspecific level (this has been previously discussed within Section 2.1). However, it may be that established interspecific relationships between egg size and organic content are too crude to retain validity on application to intraspecific studies. Indeed, Vance (1974) considered the application of trends obtained through interspecific studies to be irrelevant in the consideration of intraspecific relationships between egg size and later life history parameters.

At the intraspecific level, evidence within the literature for a relationship between egg size (diameter or volume) and organic (or energetic) content is apparent within both the vertebrates and invertebrates. For example, amongst the Vertebrata egg size has been reported to exhibit a high level of correlation with organic content in eggs of the stream fish *Poeciliopsis* sp. (Quattro & Weeks, 1991) and amongst salamanders (Kaplan, 1980a & b). Among the marine invertebrates however, empirical evidence of the putative relationship between

egg size and organic content is somewhat more equivocal. For example, an intraspecific comparison of egg size and organic content in *Solaster stimpsoni* (mean egg diameter 0.400mm) by McEdward & Carson (1987) demonstrated that egg size was indeed linked to organic content to a significant degree within this species. However, the observed relationship was not considered a sufficiently reliable predictor of egg quality (and thus energetic investment) to be applied with confidence in the consideration of life history studies. Similarly, McEdward & Coulter (1987) made a quantitative assessment of egg size and organic content in another echinoderm, *Pteraster tessellatus*, and found that egg size and volume did not necessarily correspond to egg organic content.

In contrast, a significant relationship between egg size and energetic content was established in the isopod *Ceratocerolis trilobitoides* by Clarke & Gore (1992). Egg size was also found to be a reliable indicator of egg energetic content within the Caridean polar shrimps *Chorismus antarticus*, *Notocrangon antarticus* and *Eualus gaimardii* (Clarke, 1993). All criteria by which egg content was determined - those of organic matter, dry weight, and carbon and nitrogen content - showed a significant relationship with egg volume. This study by Clarke (1993) was distinct from several previous studies, not only in establishing a significant correlation between egg size and organic content, but also in the use of clutch mean values for analysis. In previous studies performed upon on echinoderm species by both McEdward & Carson (1987) and McEdward & Coulter (1987) in which no relationship between egg size and organic content was found, analysis was performed at the individual egg level. In addition, whilst the study of McEdward & Carson (1987) included 25 females, that of McEdward & Coulter (1987) employed the progeny of just one. Therefore the absence of a significant correlation between egg size and organic content in several intraspecific studies may possibly be attributable, to a greater or lesser degree, to facets of the experimental protocol employed rather than the absence of any relationship between egg size and organic content.

Perhaps the study most relevant, and applicable, to the present investigation is that of Baur (1994). It is notably the only published example of an intraspecific quantitative evaluation of the relationship between gastropod egg size and organic content. Baur (1994) assessed both within- and among-clutch variation in egg volume, organic weight, nitrogen and carbon content in *Arianta arbustorum*. Within-clutch variation in all parameters was significantly less than between-clutch variation, indicating that *A. arbustorum* produces progeny of relatively consistent

size and organic content within clutches compared to the variation observed between clutches. This observed equal investment in progeny within clutches may be interpreted to provide further support for the models of Gadgil & Solbrig (1972) and Smith & Fretwell (1974). A significant relationship between individual egg volume and both carbon and nitrogen content was established in only 23/36 and 22/36 clutches respectively, whilst mean clutch egg volume increased significantly in an isometric manner with both mean clutch egg organic weight and nutrient content (C and N). The disparity between the intra- and inter-clutch relations were attributed to the greatly reduced range of egg sizes observed between eggs within the same clutch. Baur (1994) concluded that, in a similar trend to that observed among polar shrimps by Clarke (1993), mean clutch egg size scaled with nutrient content and could be considered to be a reliable indicator of energetic investment at the between-clutch level.

The studies of Baur (1994) and Clarke (1993) would offer support for the view that the relationship between egg size and organic content may be inferred with a greater degree of confidence at the between-clutch level rather than at the level of the individual egg. Indeed, the study of Baur (1994) constitutes quantitative data for the most taxonomically proximate species to *Adalaria proxima*, and demonstrates that, at least in some species of gastropod, egg size may be regarded as a reliable indicator of *per capita* energetic investment at the interclutch and population level. It is this between-clutch level which is employed within the present study - all egg size and subsequent selected life-history parameters (duration of the embryonic period, hatching success and larval shell length) were determined at the mean clutch, and not the individual progeny level. Whilst a direct determination of organic content in *Adalaria proxima* eggs therefore may have been optimal, the use of clutch and population mean values in preference to individual egg data may confer a greater degree of confidence to the present findings.

### Embryonic Development Time

Vance (1973a, b) constructed a theoretical model proposing a positive correlation between egg organic content (and, by inference, egg diameter) and length of the prefeeding period among marine invertebrates (see Section 2.1). In addition, several subsequent comprehensive studies based upon empirically derived data have demonstrated the presence of such a relationship among the



Opisthobranchia. The first of these was supplied by Thompson & Jarman (1986) using data obtained for 60 species from the Atlantic and Pacific Oceans. The authors concluded embryonic period to be affected by both the temperature at which spawn masses were reared, and by ovum diameter. An increase in diameter from 73 $\mu$ m to 181 $\mu$ m increased the duration of the embryonic period by a factor of 2.02. The authors attributed the greater embryonic period shown by larger eggs to developmental retardation caused by larger quantities of yolk within the egg. In a comprehensive review of developmental modes within the Opisthobranchia Hadfield & Miller (1987) observed a similar trend of longer embryonic periods in species producing larger eggs. However, whilst these studies may be informative on an interspecific level, such rudimentary comparisons among taxa may not be valid when considering the more subtle relationship between egg size and development time evident within a species (Vance, 1974).

The mean population embryonic periods (time period from laying to when >50% of larvae hatched) observed within the present study varied significantly between populations. Despite this, there appeared to be no correlation with mean population egg volume, providing further evidence of the invalidity of interspecific models on application to intraspecific studies.

The provenance of *Adalaria proxima* spawn masses utilized in order to determine embryonic period in previous studies has not been considered as a possible cause of variation. Thompson (1958) reported embryonic periods of between 57-60d at 5.2°C (n=4) and 45-47d at 7.0-7.7°C in spawn masses originating from the Menai Straits, Anglesey, whilst Todd & Havenhand (1985) reported greater embryonic periods of 68.0d at 6°C (n=2) in spawn masses produced by adults taken from Seil Island, Argyll. These differences may be explained by provenance; interpopulation means established within the present study show the same relative trend - the population mean of 63.9d  $\pm$  0.9 (n=31) in spawn masses associated with Menai Bridge is significantly lower than that of Cuan Ferry, Seil Island which has a mean embryonic period of 71.0d  $\pm$  1.0 (n=40).

The source of the considerable interpopulation differences in embryonic period established within the present study was investigated by examining the characteristics of intrapopulation embryonic period data. Although the majority of observed variation in embryonic period was attributable to the population effect ( $P < 0.001$ ), a significant proportion of total variation was evident between

individuals within populations ( $P < 0.05$ ). In contrast to the previous reproductive parameters examined (egg size and number) the duration of spawn mass embryonic period was not established to be affected by position in the spawn sequence ( $P < 0.25$ ). In addition, embryonic period showed, at best, a tenuous link with mean spawn mass egg size within populations, a significant correlation (at the  $P < 0.05$  level) being apparent in only one of five populations (that of Cuan Ferry). In a similar manner, Kaplan (1980a) found that egg size did not affect the time to hatching within three species of salamanders (*Ambystoma tigrinum*, *Ambystoma maculatum* and *Ambystoma opacum*). In contrast, Rossiter (1991) found that egg size in the gypsy moth *Lymantria dispar* did have a significant effect upon time to hatching.

Within the present study reproducing adults were subject to increasing ambient water temperatures ranging between 5.4°C (January) and 10.0°C (May) over the course of the spawning season. All populations showed a trend of increasing embryonic period with raising water temperature in the 28d immediately prior to spawning. Indeed, a significant positive relationship between these two factors was established within four of the six populations studied. Such a relationship is counter-intuitive and obviously contrary to the accepted trend of decreasing developmental times with increasing temperature reported within the literature (Thompson, 1958; Scheltema, 1967; Landry, 1975; Byrne *et al.*, 1978; Pechenik, 1984; Lima & Pechenik, 1985; Todd & Havenhand, 1985; Pechenik, 1987; Zimmerman & Pechenik, 1991). During the present study, spawn masses were isolated and incubated at a uniform temperature of 6°C, irrespective of the temperature regime under which they were laid. As mean water temperature to which the adult had been subjected increased therefore, the disparity between adult temperature regime and the spawn mass culture temperature would have increased. This may account for the increasingly retarded developmental rates observed at 6°C over the course of the spawning season. Thus, the increase in embryonic period with increasing mean water temperature, rather than being the expression of an ecologically significant developmental parameter, may more probably be considered an experimental artefact. This would be consistent with the model formulated by Havenhand (1993) who considered the significance of variation in the length of the egg-to-juvenile period (EJP) in *Adalaria proxima* in relation to lineage fitness. The author constructed a conceptual model relating the EJP to both size at reproduction and generation time. Using an EJP of 34d (at 10°C) Havenhand (1993) concluded that variation in the EJP (and, by inference the embryonic period) would not result

in large changes in subsequent fitness. The embryological data do however intimate that the temperature regime to which adults are subjected in the four weeks prior to spawning may potentially result in embryos adapted to differing optimal developmental temperatures. Any such adaptive response to a temporally varying environmental parameter (such as water temperature) may only be possible if the adult can assess the conditions and respond appropriately (McGinley *et al.* 1987). In addition, such a response will only be of advantage if the conditions encountered by the parent are a reliable predictor of the conditions which will be encountered by the progeny (Brody & Lawlor, 1984).

Thompson (1958) investigated the developmental physiology of *Adalaria proxima* by manipulating culture temperatures. Adults and spawn masses were raised at one of five set temperature regimes between 5°C and 18.0°C. The optimal reproductive range was considered to lie within the 6°C to 11°C range, below 5°C eggs appeared infertile. Most notably, spawn masses laid at 9°C-10°C could be successfully transferred from 10°C down to 5°C (although no information is available on the resultant increase in embryonic period shown by eggs laid at 10°C). However, spawn masses laid at 5.0°C could survive transplantation to neither 7.0°C-7.7°C or 9.0°C-10.0°C. Therefore, whilst eggs from adults acclimated to 10°C could withstand a drop of 5°C, the converse was not true - indeed, eggs laid by adults acclimated to 5°C could not even withstand an increase of 2°C. The present study provides evidence for the adaptation of developmental processes to the predictable seasonal increase in water temperature which occurs over the spawning season. Such physiological adaptation of later-laid eggs to elevated water temperatures may be of considerable adaptive significance in *A. proxima* by allowing the reproductive period to extend over a period of several months encompassing an increase in water temperature from 5°C - 10°C.

### Hatching Success

The spawn masses produced by six populations of *Adalaria proxima* within this study were found to display significant differences in mean hatching success at the interpopulation level, although no correlation between egg size and hatching success was established at this level.

The most notable feature apparent at the intrapopulation level was the considerable degree of variation in hatching success observed between spawn



masses. This variation was not found to be attributable to any significant degree to between-individual differences in the hatching success of spawn masses ( $P > 0.25$ ). Rather, a significant proportion of the observed variation was explained by spawn mass position in the spawn sequence ( $P < 0.025$ ). Within every population the hatching success of spawn masses appeared to decrease rapidly with increasing position in the spawn sequence. The most successful spawn masses were consistently those laid first or second by adults within all populations. Hatching success was observed to decline with increasing position in the spawn sequence to such a degree that those laid sixth or later rarely produced any viable larvae at all. This beggars the question of whether the spawning season would continue so long, and therefore so many spawn masses be produced, in the field. It is possible that the protected environment offered by the laboratory allows an extension of adult life relative to life expectancy in the field. However, if the levels of hatching success obtained within the laboratory are reflective of natural field levels then the relative importance of the genetic input of later spawn masses to the next generation would appear minimal.

#### Hatchling Size and Survival

In an extensive interspecific survey of developmental characteristics within the opisthobranch molluscs, Hadfield & Miller (1987) found that within both the Opisthobranchia, and more particularly the Nudibranchia, the mean egg size of a species could be considered a reliable predictor of shell size at hatching.

Larval shell length varied significantly between populations by as much as 10% in the present study. It may be intuitive to expect intraspecific differences in egg size to affect larval size, and indeed, smaller eggs were established to result in smaller larvae. For example, with the exception of Kinkell Braes (for which larval shell length was obtained for only three spawn masses), the ranking of mean egg volume amongst populations correlates with that of mean shell length. In addition, spawn mass mean zygote diameter was shown to display a significant relationship with mean spawn mass larval shell length. Therefore both at the population and individual spawn mass level egg size may be considered a reliable indicator of larval shell size in *Adalaria proxima*. Although the intraspecific findings of this study conform to the predictions derived from the interspecific survey of Miller &

Hadfield (1987) there are also many other examples of linkage between egg size and larval size at the intraspecific level.

For example, within the Vertebrata, Blaxter & Hempel (1963) found that larval size was proportional egg size in the herring (*Clupea harengus*). Larger ova were also found to result in larger larvae in three species of salamanders (*Ambystoma* sp.) studied by Kaplan (1980a). Further, the latter author found the difference in absolute larval sizes to be amplified after feeding began, and concluded that within these species the adaptive significance of intraspecific variation in egg size (and thus larval size) occurred only after feeding was initiated.

Within marine invertebrates, much study concerning egg size and the size and survival rates of resulting larvae has been performed upon the bivalves *Mercenaria mercenaria* (the hard clam) and *Argopecten irradians* (the bay scallop). Day (1979) established that large *M. mercenaria* larvae resulting from larger eggs set sooner and subsequently display greater growth rates than do smaller siblings. Within both *M. mercenaria* and *A. irradians*, larger eggs are associated with significantly higher levels of short term (48hrs) and longer term (1 month) larval survivorship (Kraeuter *et al.*, 1982). This may be attributed to the larger energetic reserves available for development within larger bivalve eggs (Bayne, 1972; Bayne *et al.*, 1975). Elevated larval survival rates in larger larvae have also been reported in the freshwater prawn *Palaemon paucidens* (Mashiko, 1985). Within the Gastropoda, greater survival prospects have also been attributed to larger offspring of the land snail *Arianta arbustorum* (Baur, 1990).

### Conclusion

This study demonstrates that populations of *Adalaria proxima* display significant variation in the allocation of reproductive effort to both spawn mass egg number and egg size over the eight U.K. sites surveyed. Fecundity was not found to be related to egg size at the interpopulation level, neither did the observed interpopulation differences between egg size and number exhibit a discernible latitudinal cline. The findings of this study demonstrate the importance of considering the provenance of experimental subjects. Whilst the possibility of random genetic drift cannot be excluded (Todd *et al.*, 1994), the observed interpopulation variation in division of reproductive resource may be the resultant of

differential selective forces arising from distinctive environmental conditions acting between sites.

Considerable variation in spawn mass egg number and egg size was apparent within all six populations surveyed in detail. This was not found to be attributable to between-individual variation in egg size. Rather, adults significantly reduced both the number and size of eggs contained within spawn masses over the course of the reproductive season. The nature of this reduction was similar within all populations, indicating that *Adalaria proxima* adults appear to compensate for the diminution of energetic reserves available for reproduction in a uniform manner. The number of eggs contained within successively laid spawn masses decreased within all populations, the largest proportion of total egg production (26-46%) being invested within the first-laid spawn masses.

The significant variation in egg size observed at the interpopulation level had no manifest effect upon hatching success. However, the hatching success of spawn masses was established to decrease significantly with increasing position in the laying sequence, such that those produced sixth or later in the sequence showed mean levels of hatching success considered to be no greater than zero. Populations were found to produce significantly different sizes of larvae at hatching within the present study. Egg size was determined to be an accurate predictor of larval size both at the inter- and intrapopulation level.

Thus, differential inter- and intrapopulation patterns of reproductive output have been established to have a discernible effect upon selected components of offspring fitness within the present study. Maximum fitness will be conveyed to those larvae resulting from earlier (first or second) laid spawn masses which contain more, and larger, eggs, display higher levels of hatching success, and result in larger larvae. Thus, the major proportion of reproductive output invested in the first-laid spawn mass may be justified by the superior fitness conferred upon resulting progeny and consequent associated competitive advantage. In contrast, the genetic contribution of successively later spawn masses to the next generation is likely to be disproportionately depressed by the lower levels of hatching success and smaller larval sizes associated with later-laid spawn masses.

## CHAPTER 3

### THE ATTAINMENT OF COMPETENCE

#### 3.1. INTRODUCTION

In order to recruit to the benthos, a planktonic larva must first become competent to settle and metamorphose in response to exogenous stimuli associated with a suitable habitat (Coon *et al.*, 1986; Morse *et al.*, 1988; Pennington & Hadfield, 1989; Bonar *et al.*, 1990; Pawlik, 1992), essential for the juvenile (Chia, 1978). This mandatory pelagic phase, during which time the larva is refractive to suitable settlement and metamorphic cues, is termed the 'precompetent' phase (Crisp, 1974; Scheltema, 1974; Hadfield, 1978a; Burke, 1983, 1986; Pechenik, 1990; Pechenik & Gee, 1993). The lecithotrophic larvae of some marine invertebrate species may attain competence within hours posthatching whilst the planktotrophic larvae of other species may spend weeks or months feeding in the plankton before attaining competency (Crisp, 1974, Scheltema, 1974; Kempf, 1981; Pechenik, 1980, 1984; Crisp, 1986, Pechenik, 1990).

Competence is defined as the ability of larvae to respond to appropriate behavioural or metamorphic stimuli (Chia, 1978; Hirata & Hadfield, 1986; Bonar *et al.*, 1990; Coon *et al.*, 1990a) and denotes the completion of those developmental changes required for the shift from the larval to juvenile modes of life (Miller & Hadfield, 1986). The terminology surrounding settlement and competence often is not clearly defined in the literature. Whilst the term 'settlement' can, in many contexts, encompass the whole shift from pelagic larvae to benthic juvenile (Hadfield, 1986; Pawlik, 1992), the attainment of competence to settle and to metamorphose are distinct processes (Bonar *et al.*, 1990; Coon *et al.*, 1990a). For example, in a study of the Pacific oyster, *Crassostrea gigas*, Coon *et al.* (1990) demonstrated by differential induction that the larval behavioural (settlement) and metamorphic pathways were discrete, the morphogenetic pathway becoming functional only after the behavioural. It is solely the attainment of metamorphic, and not behavioural, competence which lie within the confines of the present study. The developmental mechanisms involved in the onset of competency remain largely elusive, although Degnan & Morse (1995) have established the presence of a

'preparatory' developmental pathway in the abalone *Haliotis rufescens* which they propose is triggered before, or at, the attainment of metamorphic competency.

#### Intraspecific Variation In Precompetent Period

Much study concerning the onset of competence has focused on those molluscan species utilised in mariculture. The reliable prediction of competence is clearly important so that synchronous high-yield induction of metamorphosis can be attained. Whilst the duration of the obligatory pelagic period has been noted for many species, detailed studies focusing on attainment of competence and variables affecting its timing have been relatively fewer in the literature. Notable exceptions include the bivalves *Crassostrea gigas* (Bonar *et al.*, 1990; Coon *et al.*, 1990a) and *C. virginica* (Bonar *et al.*, 1990), and amongst the gastropods, the prosobranchs *Crepidula plana* (Zimmermann & Pechenik, 1993) and *C. fornicata* (Pechenik & Heyman, 1987; Pechenik & Gee, 1993), and the nudibranch *Phestilla sibogae* (Hadfield, 1984).

Although the onset of competence is regarded as being species specific (Chia, 1978) there may be appreciable variation in metamorphic attainment among even sibling progeny (Hadfield, 1977, 1978a; 1984; Hadfield & Scheuer, 1985; Hubbard, 1988; Bonar *et al.*, 1990; Coon *et al.*, 1990a; Todd *et al.*, 1991; Haws & DiMichele, 1993). Such variability in attainment of competence has previously been reported for *Adalaria proxima* by Todd *et al.* (1991). In the first major work documenting the nudibranch *A. proxima*, Thompson (1958a) reported the pelagic lecithotrophic larvae to undergo a precompetent period of between one and two days. Subsequent studies by Todd and co-workers, although seemingly confirming a 24-48 hr obligatory phase (Todd *et al.*, 1991), reported variability in the length of the precompetent phase from observations of occasional immediate post-hatching metamorphosis (Todd *et al.*, 1989).

The genetic basis for such variation in the attainment of competence was investigated by Hadfield (1984) using the tropical nudibranch *Phestilla sibogae*. Selective inbreeding for early onset of competence was performed over 27 generations in the laboratory, yet the ultimate resultant progeny still retained high variation in age at competence. Whilst homogeneity may, to some degree, have been compromised by the hermaphroditic sexuality and sperm storage capacity of



*P.sibogae*, Hadfield concluded that the between-sibling variation in attainment of competence could not be attributed to straightforward genetic processes.

The causes of this observed variability in attainment of competence were considered by Havenhand (1991) in his review of the behaviour of opisthobranch larvae. The first of two explanations postulated by Havenhand assumed synchronous embryonic development within a spawn mass and that larvae which have undergone eclosion (hatching from the primary capsule) may be physically impeded by the mechanical properties of the spawn mass from hatching (leaving the spawn mass). Both within Havenhand's review and this present study 'eclosion' is classed as an embryological occurrence and defined as larval emergence from the primary egg capsule within the spawn mass. This is distinct from the term 'hatching', which refers to subsequent escape of a larva from the spawn mass body.

The strength and morphology of egg masses and enclosed primary capsules varies greatly within the Gastropoda (Perron, 1981; Pechenik, 1982, 1986). Within the Opisthobranchia spawn masses consist of primary egg capsules containing embryos encased in a gelatinous matrix or stroma (Thompson, 1976; Strathmann & Chaffee, 1984; Eyster, 1986), the whole being attached to the substratum (Todd, 1985). This spawn mass structure has been suggested to offer protection to the developing embryos (Perron, 1981; Todd, 1981; Eyster, 1986; Strathmann & Strathmann, 1989), from predation (Pechenik, 1979; Todd, 1985), desiccation to a limited degree (Pechenik, 1978), osmotic stress (Pechenik, 1982, 1983), physical damage (Perron, 1981) and possible pollutant stress (Todd, 1981). In a study of the relative protection afforded by egg masses within the Prosobranch genus *Conus* spp., Perron (1981) found that resistance to mechanical injury was higher in species possessing a relatively longer embryonic development period. The employment of 'stronger' or 'tougher' spawn masses by species which undergo prolonged period of intracapsular development is also apparent within the Opisthobranchia (Havenhand, 1991). For example, spawn masses containing embryos requiring a longer embryonic phase, such as lecithotrophs, must necessarily remain intact for an absolutely and relatively greater period than those undergoing planktotrophic development (Pechenik, 1986a) and will consequently lose integrity at a slower rate than the spawn masses of those species employing planktotrophic larvae (Havenhand, 1991). For example, the spawn mass of *Adalaria proxima* is relatively more robust than that of the related planktotroph *Onchidoris muricata* (Todd, 1979; Havenhand, 1981), but also is relatively less

substantial than that of the non-pelagic lecithotroph (direct developer) *Cadlina laevis* (Havenhand, 1991).

Confinement of eclosed veligers by the exterior spawn mass membrane may be exacerbated by any differential degradation of stroma. For example, bacterial action (Harris, 1975) or mechanical attrition may cause the outer portion of the stroma to lose integrity prior to the more central portion, thus resulting in differential hatching within a spawn mass. Whilst larvae will be developmentally synchronised, those more peripherally sited larvae will be physically able to liberate themselves faster than relatively more centrally placed siblings. Trapped veliger larvae may therefore spend a proportion of the precompetent period within the confines of the spawn mass membrane (Havenhand, 1991). The observed presence of active eclosed *A. proxima* veligers held within the confines of the spawn mass (Havenhand & Todd, unpubl. obs. in Havenhand, 1991) may infer that the reported variability in onset of competence in this species is attributable, at least in some degree, to physical impediment of larval hatching rather than any asynchrony in development.

An alternative, although not mutually exclusive, hypothesis put forward by Havenhand (1991) to explain observed variability in attainment of competence, concerns ontogenetic heterogeneity within a spawn mass due to restricted gaseous exchange. Differential development within large piscine spawn masses is known to occur as a result of reduced gaseous exchange; for example, retardation of central embryos has been documented in spawn masses of the cod, *Ophiodon elongata* (Giorgi & Congleton, 1984). Developing embryos are dependent on the process of diffusion for the supply of oxygen and removal of waste products, the rate of diffusion being proportional to the thickness of the gelatinous layer (Strathmann & Chaffee, 1984). Embryos centrally situated within a spherical or tubular spawn mass will be subjected to less efficient gaseous exchange than those sited nearer the spawn mass-seawater interface, and consequently may develop at a relatively slower rate (Chaffee & Strathmann, 1984; Strathmann & Chaffee, 1984). Direct experimental evidence of this effect was achieved by the manipulation of artificially constructed spawn masses by Strathmann & Strathmann (1989). Monitoring of the ontogenetic progress of planktotrophic echinoid eggs enclosed in an agarose matrix revealed that the developmental rate of central embryos was compromised by overcrowding of eggs and/or thickness of the spawn mass. Evidence of this contrived effect occurring in the natural state was supplied by the observations of Chaffee & Strathmann (1984) on the lobed gelatinous spawn mass of the



polychaete *Nereis vexillosa*. The metabolic rate of embryos, therefore, may be directly influenced by the morphology of the spawn mass and developmental rate restricted by sub-optimal oxygen concentrations (Eyster, 1986; Pechenik, 1986a; Strathmann & Chaffee, 1984) or reduced removal of waste products by diffusion (Eyster, 1986). Such an example within the Opisthobranchia derives from a study of the gelatinous spawn masses of the cephalaspidean *Melanochlamys diomedea* by Chaffee & Strathmann (1984). A pattern of retarded development of centrally placed embryos relative to peripheral embryos was established, which implied that this asynchrony could be attributed to restricted gaseous exchange. Thus, when development rates within a spawn mass are differentially affected by oxygen supply, hatching will not be synchronous, and whilst all larvae will undergo an obligatory precompetent phase on hatching, the onset of competence between siblings will be staggered (Havenhand, 1991).

#### Determination of competence

The accurate determination of the onset of competence is of importance in defining the termination of the precompetent phase. This precompetent period constitutes the minimum pelagic period of the larva (Chia, 1978a, Scheltema, 1978) and is therefore relevant when determining the capacity of a species to delay metamorphosis (Pechenik & Gee, 1993). The attainment of competence may often be accompanied by diagnostic morphological changes (Chia, 1978; Hadfield, 1978), such as the appearance of the propodium in the lecithotrophic larvae of the nudibranch *Phestilla sibogae* (Bonar & Hadfield, 1974). Competence is indicated in the planktotrophic veligers of the dorid nudibranch *Hypselodoris infucata* not only by the development of the propodium such that its length exceeds the diameter of the operculum, but also by a host of other anatomical features. The mantle retracts from the shell margin, lipid droplets are visibly discernible within the digestive gland and the architecture of the larval shell alters to produce a small lip fringing the margin (Hubbard, 1988).

Morphological features, size, behaviour and age since hatching are not however, necessarily, reliable reflections of larval physiological state (Pechenik & Heyman, 1987; Zimmermann & Pechenik, 1991; Haws & DiMichele, 1993). In a study of the ontogenetic appearance of competence in the larvae of *Crassostrea gigas* and *C. virginica*, Bonar *et al.* (1990) established a correlation between competence and morphology (shell size and presence of eye spots). However,

sufficient variation between the onset of competence and larval morphology was observed for the authors to conclude that any such determination of competence should be viewed with caution. Indeed, this was confirmed by Haws & DiMichele (1993) who established that the onset of competence relative to the presence of larval eye spots and development of the pedal organ varied by several days in a significant number of cases. Larval shell length and morphology have been established to be unreliable indicators of physiological status in studies of the gastropods *Crepidula plana* (Zimmermann & Pechenik, 1991) and *C. fornicata* (Pechenik & Heyman, 1987). In their investigation of environmental conditions on growth and the onset of competence in *C. plana*, Zimmerman & Pechenik (1991) established that whilst competent larvae were significantly larger than precompetent larvae, shell length was not a reliable diagnostic feature in the prediction of competence. Moreover, considerable intraspecific variation in larval morphology (appearance of gill filaments and shell brims) was apparent at competence and the length of the precompetent period was affected by environmental conditions such as water temperature and salinity.

Whilst *Adalaria proxima* does possess a propodium (Thompson, 1958a) akin to that of *Phestilla sibogae* (Bonar & Hadfield, 1974), there are no apparent definitive diagnostic morphological developments commensurate with the onset of metamorphic competence. As a consequence of the unreliable reflection of physiological state by morphological and behavioural attributes, competence may, in many species, be defined solely by the successful initiation of metamorphosis. In addition to appropriate natural cues, within the laboratory a range of artificial morphogenic agents may be utilized.

### Induction of Metamorphosis

*Adalaria proxima* may be successfully induced to metamorphose in response to the primary adult prey, the cheilostome bryozoan *Electra pilosa* (Thompson, 1958a, Todd *et al.*, 1991; Lambert & Todd, 1994). That the cue could be water-borne was first proposed by Thompson (1958a) and later confirmed in a series of experiments by Lambert & Todd (1994); see Chapter 4 for further discussion.

The chemical structure of natural metamorphic inducers for the vast majority of marine invertebrate larvae are as yet generally unknown (see Pawlik, 1992 and Chapter 4 for a discussion of this). The acquisition and administration of known

concentrations of natural inducer such that a predictable response may be induced within the laboratory or commercial culture facility is, therefore, a difficulty (Pechenik & Heyman, 1987; Eyster & Pechenik, 1988; Hadfield & Pennington, 1989; Pechenik & Gee, 1993). The study of larval mechanisms active in the processes of attainment of competence, settlement and metamorphosis are therefore facilitated by the use of readily available and quantifiable artificial induction agents allowing the predictable and repeatable induction of high levels of metamorphosis (Yool *et al.*, 1986; Eyster & Pechenik, 1988; Pechenik & Gee, 1993). The induction of metamorphosis in response to artificial substances has been thoroughly reviewed (Hadfield, 1978a; Pawlik, 1990, 1992; Rodriguez *et al.*, 1993). These artificial cues may be categorised according to their nature, and include organic solvents, inorganic ions, and neuroactive substances. The specific modes of action by which these artificial morphogenic agent are proposed to act are addressed within Section 3.4 (Discussion).

### Inorganic Ions

Subjection of larvae to elevated concentrations of inorganic ions (chiefly monovalent cations) in the surrounding sea water medium have been found to be capable of eliciting responses in many marine invertebrate groups. The standard salt composition of artificial sea water, which may be manipulated by the addition of salts to give known elevated concentrations of any cation, is detailed by Baloun & Morse (1984). This class of cues includes the ammonium ion ( $\text{NH}_4^+$ ), potassium ( $\text{K}^+$ ), caesium ( $\text{Cs}^+$ ) and lithium ( $\text{Li}^+$ ). Larvae of the hydroid *Hydractinia echinata* were successfully triggered to settle and metamorphose to the polyp stage by Berking (1988) using a range of cations ( $\text{Li}^+$ ,  $\text{Rb}^+$ ,  $\text{Ba}^+$  and  $\text{NH}_4^+$ ). Larvae of the ascidian *Ciona intestinalis* have been demonstrated to metamorphose in response to excess (above ambient) concentrations of  $\text{NH}_4^+$ ,  $\text{Cs}^+$  and  $\text{Li}^+$  (Berking & Herrmann, 1990). The potential metamorphic activity of several mono- and divalent cations was assessed by Pechenik & Heyman (1987) using the prosobranch gastropod *Crepidula fornicata*. These authors found that both  $\text{Na}^+$  and  $\text{Ca}^{2+}$  were ineffective over a range of concentrations (10-20mM excess) in inducing metamorphosis. Whilst  $\text{Rb}^+$  and  $\text{Cs}^+$  did induce high levels of metamorphosis, the potential usefulness of these cations as general artificial inducers was negated by severe toxic effects, manifest by the moribund state and subsequent total mortality of resulting juveniles. Whilst  $\text{Cs}^+$  was a successful trigger for *C. fornicata* larvae

(Pechenik & Heyman, 1987), neither  $\text{Cs}^+$  or  $\text{Li}^+$  induced settlement or metamorphosis in another prosobranch gastropod, *Haliotis rufescens*; indeed, both cations proved to be toxic at less than 9mM excess (Baloun & Morse, 1984).

Potassium is inductively the most widely effective, and therefore extensively cited, of the cations in the literature. Elevated potassium levels have been utilised to successfully trigger both settlement and metamorphosis in a spectrum of marine invertebrate larvae, including polychaetes (Yool *et al.*, 1986; Cameron *et al.*, 1989), gastropods (Baloun & Morse, 1984; Trapido-Rosenthal & Morse, 1985; Yool *et al.*, 1986; Pechenik & Heyman, 1987; Eyster & Pechenik, 1988; Hubbard, 1988; Coon *et al.*, 1990; Todd *et al.*, 1991), and echinoderms (Pearce & Scheibling, 1994). The non-specific nature of elevated potassium is well illustrated by the study of Yool *et al.* (1986) in which the inductive property of potassium was tested using three species of gastropod mollusc (*Phestilla sibogae*, *Astraea undosa* and *Haliotis rufescens*) and an annelid polychaete (*Phragmatopoma californica*). Each of these species occupy differing habitats, all have been demonstrated to display specificity in relation to their natural inductive cues (*P.sibogae*, Hadfield [1984], *A.undosa* Morse *et al.* [1980] and *H.rufescens*, Morse *et al.* [1984] *P.californica* Jensen & Morse [1984]), and yet all four were induced to metamorphose in response to elevated potassium, albeit at different concentrations.

Several species of prosobranch gastropod have been demonstrated to be sensitive to increased concentrations of potassium ions. For example, in examining the role of excitable cells in the response of the nervous system to metamorphic stimuli, Baloun & Morse (1984) successfully induced metamorphosis in larvae of the red abalone *Haliotis rufescens* using elevated potassium. They found that an exposure time of at least 20 hours was required, and subsequent settlement and metamorphosis were induced in a dose dependent manner. Further studies by Trapido-Rosenthal & Morse (1985) and Yool *et al.* (1986) confirmed the sensitivity of *H.rufescens* to elevated potassium levels.

Elevated levels of potassium were also found to be an effective and reliable method of inducing metamorphosis of veliger larvae of the prosobranch *Crepidula fornicata* (Pechenik & Heymann, 1987). From a range of 5-50mM excess  $\text{K}^+$  test solutions the optimal dose was determined to be 20mM. Although metamorphosis could be induced within 5 hours of treatment initiation, for successful induction to proceed exposure was required to be continuous. A later study (Eyster & Pechenik,



1988) extended this investigation, and by inducing metamorphosis using elevated potassium and by measuring the growth, respiration and feeding of resultant juveniles, they determined that metamorphic induction by elevated potassium had no significant negative effects. Elevated potassium also has proved successful in another prosobranch, *A.undosa*. Administration of the optimal dose (10mM excess) induced a rapid initiation of settlement, followed by metamorphosis (Yool *et al.*, 1986).

Subjection of veliger larvae to excess potassium has also proved effective in inducing metamorphosis among several species of opisthobranch gastropods (Hubbard, 1988; Yool *et al.*, 1986; Todd *et al.*, 1991). Planktotrophic larvae of the tropical dorid nudibranch *Hypselodoris infucata*, for example, have been demonstrated to metamorphose in response to 10-30mM elevated potassium, although 20mM was considered optimal (Hubbard, 1988). Mean percent larval responses were less than those elicited by the natural cue (a range of sponges) but the results were not considered statistically significantly different when compared for each day post-hatching. Thus Hubbard concluded that elevated potassium could be regarded as a reliable indicator of competence in this species. In addition, Yool *et al.* (1986) found that the lecithotrophic larvae of the tropical nudibranch *Phestilla siboga* were induced to metamorphose (in a dose dependent manner) by an excess potassium level of 20mM. A response of 70% metamorphosis after 72h exposure showed elevated potassium to be a highly effective metamorphic cue.

The metamorphic induction capacity of elevated potassium on *Adalaria proxima* has been comprehensively investigated by Todd *et al.* (1991). Potassium concentrations up to 30mM excess were assayed for morphogenic activity. *A.proxima* veliger larvae were found to be sensitive to a rise of just 2.5mM above usual sea water potassium content. In common with several other species of gastropod (Yool *et al.*, 1986), the optimal concentration, capable of inducing a mean 77% metamorphosis, was demonstrated to be 10mM excess. Supra-optimal concentrations above, 19mM excess were less effective or inhibitory, as characterized by larval inactivity and retraction into the shell. A series of timed exposure experiments revealed that, in common with other gastropods (Pechenik & Heyman, 1987; Hubbard, 1988), continuous exposure to the cue was required.

Sensitivity to elevated potassium concentration has been established to vary interspecifically, and where the optimal dose is exceeded, toxic effects become

apparent. For example, whilst both the echinoids *Strongylocentrotus droebachiensis* and *Echinarachnius parma* are triggered to metamorphose in a dose dependent manner to elevated potassium ion concentration, concentrations below 80mM were ineffective for the former species, yet toxic to the later (Pearce & Scheibling, 1994). The optimal dose for *S. droebachiensis*, triggering 92% metamorphosis, was 100mM, yet for *E. parma* the optimal dose was 40mM (92% metamorphosis induced). Similarly, amongst the gastropods, *Phestilla sibogae* and *Crepidula fornicata* are optimally induced to metamorphose by a continuous dose of 20mM excess potassium (Yool *et al.*, 1986; Pechenik & Heyman, 1987), whilst for *Haliotis rufescens* and *Adalaria proxima* this is supra-optimal, the optimal dose being just 10mM excess (Yool *et al.*, 1986; Todd *et al.*, 1991).

Whilst elevated potassium concentration is perhaps the most successful and widely applicable of the monovalent cations, it is not an effective inducer of invertebrate metamorphosis in all species. Such is the case for the larvae of the echinoids *Acanthaster planci* (Johnson *et al.*, 1991) and *Strongylocentrotus purpuratus* (Rowley, 1989). Competent *A. planci* (crown-of-thorns starfish) larvae subjected to elevated potassium treatments spanning the range of 10-40 mM excess were neither triggered to settle nor to metamorphose. The sole response was a temporary retraction of larval arms at 40 mM K<sup>+</sup>, apparently due to the toxic nature of the treatment (Johnson *et al.*, 1991). Examples of potassium insensitive species are not, however, confined to the echinoderms: Morse *et al.* (1988) found larvae of the anthozoan *Agaricia* spp. to be unaffected by elevated potassium. Similarly, from the mollusca, excess potassium proved ineffective when administered to the bivalve *Mytilus edulis* (Eyster & Pechenik, 1987).

The possibility that metamorphic induction is mediated not by an increase in potassium ions *per se*, but rather, by increased osmolarity of the test solution resulting from the addition of KCl and subsequent dissociation into both K<sup>+</sup> and Cl<sup>-</sup> ions, has been addressed by several authors. Baloun & Morse (1984) presented evidence that it was the external potassium concentration which was responsible for the induction of metamorphosis in *H. rufescens* larvae by utilizing artificial seawater to maintain constant osmotic pressure by ion replacement. This, and studies by other authors, confirmed that metamorphosis could be attributed to the ionic species administered and not elevated osmolarity (Yool *et al.*, 1986; Pechenik & Heyman, 1987; Cameron *et al.*, 1989; Todd *et al.*, 1991). Evidence from screening of other monovalent cations for metamorphic inducing capacity (Baloun & Morse, 1984; Pechenik & Heyman, 1987) offers further support for the activity

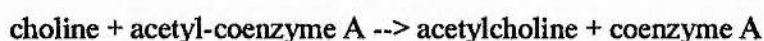
of the potassium ion itself. Quantification of the increase in osmotic pressure caused by addition of cations by Pechenik & Heyman (1987) established that, from a starting point of  $932 \text{ mOsm}\cdot\text{l}^{-1}$  in control seawater, osmolarity was raised a mere  $18 \text{ mOsm}\cdot\text{l}^{-1}$  (<2%) in test solutions, an effectively negligible rise. This confirmed the validity of experiments utilizing the protocol of ion supplementation in preference to ion replacement by manipulation of artificial seawater (Baloun & Morse, 1984).

### Neuroactive Compounds

Many marine invertebrate larvae may be induced to metamorphose, with varying degrees of success, by bioactive compounds (Pawlik, 1990). This class of artificial inducers includes choline and its associated derivatives- catecholamines such as dopamine and epinephrine (adrenalin)- and other amino acid derivatives such as GABA .

#### i. Choline

Choline (ethylol-trimethyl-ammonia hydrate), may be present in several forms within animal tissue. Combined with glyceryl-phosphoric acid it forms lecithin, a constituent of cellular membranes in both vertebrate and invertebrates (Blusztajn & Wurtman, 1983; Pawlik, 1990). It is, however, the free form within cholinergic neurones that is of interest when considering neuronal control of metamorphosis, because here choline is the precursor of the neurotransmitter acetylcholine (Blusztajn & Wurtman, 1983; Pawlik, 1990). Synthesis of acetylcholine is catalysed by choline acetyltransferase and proceeds by the reaction:



(Blusztajn & Wurtman, 1983)

Choline, in the form of succinylcholine chloride, was initially demonstrated to induce metamorphosis in marine invertebrate larva using the opisthobranch gastropod *P. sibogae* (Bonar, 1976). Further confirmation of the capacity of choline to successfully initiate metamorphosis in this species was supplied in later studies by Hadfield (1978a, 1984) and Hirata & Hadfield (1986). An investigation of the role of succinylcholine in mediating opisthobranch metamorphosis was undertaken by Hadfield (1978a) by screening a series of 15 related compounds for morphogenic properties. The results were clear in



establishing the active agent to be not the succinyl moiety but choline. Other members of the Opisthobranchia triggered to metamorphose by choline include the nudibranchs *Eubranchius doriae* (Bahamondes-Rojas & Tardy, 1988) and *Adalaria proxima* (Todd *et al.*, 1991). By the bio-assay of a range of concentrations between  $10^{-4}$ - $10^{-1}$ M choline was found to successfully induce complete metamorphosis of *A. proxima* at  $10^{-3}$ M, with between  $5 \times 10^{-3}$ - $10^{-2}$ M being judged as the optimal concentration. Timed exposure to choline ( $10^{-2}$ M) doses established that, in contrast to elevated potassium, just 1-2 h exposure to the active agent was required for metamorphosis to proceed.

Several other invertebrates from various taxa have displayed sensitivity toward choline, although the degree of success and effective concentrations required may vary according to the form of choline applied. Larvae of the polychaete *Phragmatopoma lapidosa californica* were, for example, triggered to metamorphose in response to  $10^{-3}$ M succinylcholine, albeit abnormally in that larvae did not settle and produce juvenile tubes (Pawlik, 1990). Larvae were, however, completely unresponsive to equivalent doses of choline in other forms (choline, acetylcholine, phosphorylcholine, methacholine and carbamylcholine). Moreover, differential sensitivity to compounds was displayed at higher concentrations of inducer ( $>4.0 \cdot 10^{-2}$ M), whilst 90% of larvae metamorphosed in response to succinylcholine, the same concentration of choline elicited only 50% metamorphosis and rapid total mortality of larvae and juveniles (Pawlik, 1990). The prosobranch *Ilyanassa obsoleta* also displays this form-specific response to choline - whilst unresponsive to choline, succinylcholine and acetylcholine, acetyl-beta-methylcholine induced 80-100% metamorphosis (Leventine & Bonar, 1986).

In addition, the gastropod *Haliotis rufescens* exhibited no settlement or metamorphosis in response to treatment with choline in the form of both acetylcholine and choline (Morse *et al.*, 1979). This has reasonably been taken to indicate that, in common with other artificial metamorphic inducers, choline is not capable of initiating morphogenesis in all species. It is possible, however, that this lack of effect is merely another demonstration of form-specific response and, like *Ilyanassa obsoleta*, *H. rufescens* may be refractive to both succinylcholine and acetylcholine yet may be successfully induced to metamorphose by another choline containing compound.

## ii. GABA

GABA ( $\gamma$ -Aminobutyric acid) is a simple amino acid derivative of glutamic acid (Morse *et al.*, 1979; Pawlik, 1992) and is known to act as a neurotransmitter and neurohormone in higher animals (Morse *et al.*, 1979; Trapido-Rosenthal & Morse, 1985). The active settlement and metamorphic inducing properties of GABA and closely similar structural analogues on marine invertebrates were first demonstrated by Morse *et al.* (1979) on competent veliger larvae of the gastropod mollusc *H. rufescens*. Administration of  $10^{-7}$ - $10^{-6}$ M GABA in seawater was found to elicit settlement and metamorphosis. The attainment of competence in response to GABA and the natural cue was concurrent (6-7 days at  $15^{\circ}\text{C}$ ) and evidence of developmental metamorphosis in the form of new shell was visible 24-36 hours post exposure. Although a response was discernible using  $10^{-7}$ M GABA, the optimal dose was judged to be  $10^{-6}$ M; exposure to greater concentrations proving toxic to larvae. The structural analogy between GABA and the natural cue (associated with crustose coralline red algae), and the fact that both occur in a conjugated form with proteins and other macromolecules, led the authors to link GABA to the natural settlement and metamorphic cue (Morse *et al.*, 1979; Morse & Morse, 1984b). Subsequent studies by Morse and co-workers investigating the properties and identity of the natural cue substance have confirmed the inductive properties of GABA and assigned the natural cue as a 'GABA-mimetic' molecule (Morse, 1980; Morse & Morse, 1984a & b; Morse *et al.*, 1984; Baloun & Morse, 1984; Trapido-Rosenthal & Morse, 1985). The term 'GABA-mimetic' may not be the most appropriate, since little evidence for the structure or identity of the cue has, as yet, been elicited, and the structures of those few natural cues identified to date are quite unlike those of known neurotransmitters (for example see Kato *et al.*, 1975; Pawlik, 1986, 1992).

Many members of the genus *Haliotis* and several other molluscan species have since been documented to display sensitivity towards exposure to GABA (see Morse *et al.*, 1984 and references therein). The chitons *Mopalia mucosa* (Morse *et al.*, 1979) and *Katharina tunicata* (Rumrill & Cameron, 1983) produce pelagic veligers which successfully metamorphose in response to GABA. Examples are not confined to the Gastropoda; in a study of *Strongylocentrotus droebachiensis*, Pearce & Scheibling (1990b) established that  $10^{-5}$ M- $10^{-1}$ M GABA successfully induced larval metamorphosis.

Not all marine invertebrate species appear capable of responding to GABA. For example, larvae of the polychaete *Phragmatopoma lapidosa californica* showed no response to  $10^{-5}$ - $10^{-2}$ M GABA. Similarly, the soft coral *Alcyonium siderium* (Sebens, 1983) and the asteroid *Acanthaster planci* displayed complete insensitivity toward GABA (Johnson *et al.*, 1991). Assayed using a range of concentrations from  $10^{-7}$ - $10^{-3}$ M, *A. planci* larvae neither metamorphosed nor exhibited any toxic response (Johnson *et al.*, 1991). Likewise, both *Crassostrea gigas* and *Crepidula fornicata* exhibited no metamorphic response when subjected to  $10^{-6}$ - $10^{-4}$ M and  $4.0 \cdot 10^{-7}$ - $4 \times 10^{-6}$ M GABA respectively (Coon *et al.*, 1985; Pechenik & Heyman, 1987), and the gastropod *Phestilla sibogae* showed only a 'weak' response (30% metamorphosis) to  $10^{-4}$ M GABA (Hadfield, 1984). The examples above illustrate the point made by Pechenik & Heyman (1987), that GABA is a less general cue than elevated potassium.

### iii. L-DOPA and the Catecholamines

The final group of bioactive substances are derivatives of the amino acid tyrosine (2-amino-3-[4-hydroxyphenyl]propanoic acid) or occasionally peptidyl tyrosine (Waite, 1992), and consists of L-DOPA (L- $\beta$ -3,4-dihydroxyphenylalanine) and the catecholamines, a group of compounds which includes: dopamine, adrenalin (epinephrine), noradrenalin (norepinephrine), isonylalanine (IP), 3,4-dihydroxyphenylacetic acid (DOPAC) and 3,4-dihydroxymandelic acid (DOMA) (Rodriguez *et al.*, 1993; Pawlik, 1990; Pires & Hadfield, 1991).

Within invertebrates L-DOPA has functions as diverse as neuroendocrine signalling, immunity, sclerotization of egg capsules and integuments, adhesion and pigmentation (see reviews of Waite, 1992; Rodriguez *et al.*, 1993). The significance of L-DOPA as a neurotransmitter marine mediating invertebrate settlement and metamorphosis has been addressed in several studies (Jensen & Morse, 1990; Pawlik, 1990; Johnson *et al.*, 1991; Waite, 1992). Exogenously applied L-DOPA has induced metamorphosis, with varying degrees of efficacy, within several marine invertebrate taxa, namely polychaetes (Jensen & Morse, 1984; Pawlik, 1990), echinoids Burke (1983), bivalves (Coon *et al.*, 1985; Weiner *et al.*, 1985; Bonar *et al.*, 1990; Pawlik, 1990 and references therein, Chevolut *et al.*, 1991) and gastropods (Hadfield, 1984)

In a study of neural control of metamorphosis in the echinoid *Dendraster excentricus*, Burke (1983) found that the catecholamine dopamine ( $10^{-5}\text{M}$ ) induced metamorphosis to a maximum of 25% of competent larvae, whilst excised larval arms of the same species were induced to metamorphose in response to both L-DOPA and dopamine. Dopamine has also been reported to elicit limited levels of partial metamorphosis in the nudibranch *P.sibogae* (Hadfield, 1984), as is also the case for *Ilyanassa obsoleta* (Leventine & Bonar, 1987).

Larvae of the scallop, *Pecten maximus*, have been successfully induced to metamorphose in significant levels on exposure to epinephrine as well as L-DOPA (Chevolot *et al.*, 1991). In addition, the nudibranch mollusc *Phestilla sibogae* has been shown to metamorphose in response to both norepinephrine ( $10^{-3}\text{M}$ ) and epinephrine ( $10^{-4}\text{M}$ ) (Hadfield, 1984). Norepinephrine and epinephrine are not however universally applicable inducers within the mollusca: larvae of the gastropod *Ilyanassa obsoleta*, for example, have proved insensitive to the effects of both adrenalin and noradrenalin (Leventine & Bonar, 1987), as have larvae of *Haliotis rufescens* (Morse *et al.*, 1979).

### Rationale

The present study was undertaken in order to investigate the ontogenetic attainment of competence in *Adalaria proxima*. Artificial cues have been extensively utilized for the determination of competence in laboratory studies of marine invertebrate larvae (Pechenik & Heyman, 1987; Bonar *et al.*, 1990; Coon *et al.*, 1990; Pechenik & Gee, 1993; Zimmerman & Pechenik, 1993). Indeed, both the artificial cues choline chloride (5mM) and elevated potassium (19 and 29mM) have been used as positive controls in experiments investigating induction of metamorphosis and are regarded as reliable indicators of the attainment of metamorphic competence in *A.proxima* (Todd *et al.*, 1991; Lambert & Todd, 1994).

Whilst considerable variation in the precompetent period of *Adalaria proxima* has previously been documented (Todd *et al.*, 1989; Todd *et al.*, 1991), little study has investigated this phenomenon in depth. The aims of this investigation were two-fold. The initial objective was to determine the age and developmental state at which *A.proxima* larvae attained competency to metamorphose. In order to establish the earliest developmental stage at which

competence was attained unhatched late veliger stage embryos as well as hatched veliger larvae were employed. The inclusion of hatched veliger larvae also facilitated confirmation of cue morphogenic activity upon competent larvae in the event that embryos proved unresponsive.

Pechenik & Gee (1993) established that larvae of the prosobranch *Crepidula fornicata* became competent with respect to excess potassium before attaining competency towards the natural cue. This demonstration had implications for the routine laboratory study of marine invertebrate larvae by illustrating that competence may not be regarded as general, but rather, may be specific to a particular cue, thereby requiring the definition of competence to be qualified with respect to the induction agent. Moreover, comparison of the differential cue-specific attainment of competence supplied information on the active sites and mechanisms of action involved in both naturally and artificially induced metamorphosis. Supplementary to the initial objective therefore was the question : Do embryos/larvae become competent to respond to different metamorphic cues at the same time? Determination of competence was achieved using a range of metamorphic cues, each of which were expected to employ different possible mechanisms of action in triggering the initiation of metamorphosis. Those employed consisted; the known natural cue (*Electra pilosa* conditioned seawater); compounds previously established to initiate artificial metamorphosis in competent *A. proxima* larvae (elevated potassium and choline chloride); and several neuroactive agents with unknown morphogenic properties in relation to *A. proxima*, but previously demonstrated to successfully initiate metamorphosis in other marine invertebrates (GABA, L-DOPA and dopamine). Comparison of the resultant age-dependent metamorphic responses was therefore intended to elicit information concerning mechanisms of specific cue-mediated metamorphosis and ontogeny of the metamorphic pathway(s) in *A. proxima*.



## 3.2 METHODS

### Collection and Maintenance of Spawn Masses

The experiments comprising this section were performed over three annual spawning seasons. Adult *Adalaria proxima* were collected from Clachan Seil, Argyll, in May 1994, and from Kinkell Braes and Kingsbarns, Fife, during March - May 1993, 1994 and 1995. Adults were maintained, in their respective populations, in glass tanks held at approximately ambient field temperature (10°C), ambient photo period (16 hrs light, 8 hrs dark), and supplied with fresh food *ad libitum*. Spawn masses laid in the field were collected from Clachan Seil, Argyll in May 1994 and thereafter cultured within the laboratory at 10°C.

Spawn masses, which are generally laid on *Fucus serratus* in the field, and both on macroalgal surfaces and glass tank walls in the laboratory, were collected and isolated. Tanks were checked for newly spawned masses daily and the date of spawning noted to allow determination of embryo age in later experiments. Spawn masses were cultured in individual china crucibles at ambient temperature (10°C) with the entire water volume (25 ml) changed daily. At no time during the experiments were antibiotics administered. Embryonic development was monitored using a Wild-E binocular microscope and abnormal or unhealthy spawn masses discarded.

### Preparation of the Natural Cue

Natural cue treatments comprised either fresh colonies of the bryozoan *Electra pilosa* epiphytic on *F. serratus* (Experiment 4) or twice filtered sea water (TFSW) previously conditioned by *E. pilosa* (Experiments 5 and 8-13). Fresh *E. pilosa* on *F. serratus* fronds was regularly collected from Kinkell Braes, St Andrews, and Kingsbarns. Successful conditioning required the presence of active healthy *E. pilosa* colonies in TFSW at 10°C for at least 24-48 hrs prior to bioassay. Particulate material was eliminated from the resulting supernatant by filtration with a Whatman qualitative paper filter. Conditions facilitating the optimal conditioning of sea water with the appropriate metamorphic factor(s) are discussed in Chapter 4.

### Preparation of Artificial Cues

Fresh  $5 \cdot 10^{-3} \text{M}$  choline chloride (Sigma chemicals) controls were prepared for each experiment by solution in twice filtered ( $0.45 \mu\text{m}$ , thence  $0.2 \mu\text{m}$ ) sea water (TFSW). The elevated potassium treatments were obtained by simple supplementation of TFSW with potassium chloride (Sigma Chemicals) to yield final potassium ion concentrations of 19mM (Experiments 3-7 and 13) and 29mM (Experiment 5).

No information concerning the effective dose of other neuroactive agents required to induce metamorphosis in *A. proxima* was available. Consequently, dose-response curves were obtained both for GABA and dopamine over a range of concentrations documented to be effective for other gastropod species. The appropriate range for GABA was determined to be  $10^{-2}$ - $10^{-7} \text{M}$  continuous exposure. Dopamine was assayed over the concentration range  $10^{-2}$ - $10^{-5} \text{M}$ . Veliger larvae were exposed to dopamine treatments for 7 hrs, removed, rinsed in TFSW and placed in petri dishes containing only clean sea water at  $10^{\circ}\text{C}$ .

### Assignment of Embryonic Age

During culture the absolute age (in days) of each spawn mass was noted in conjunction with the incubation temperature. Environmental variables acting on an embryo from the time of spawning, however, mean that the absolute age of an embryo may not necessarily be a reliable indicator of developmental state. The embryonic period of *A. proxima* can range from 57-60 d at  $6^{\circ}\text{C}$  (Thompson 1958a) down to 36-42 d at  $9/10^{\circ}\text{C}$  (Thompson 1958a) to 30 d at  $10^{\circ}\text{C}$  (Todd & Havenhand 1985). Even within a constant temperature regime, embryonic period has been established to vary significantly between several U.K. populations of *A. proxima* (see Chapter 2). Moreover, whilst microscopic inspection within the laboratory allowed embryonic developmental state to be assigned for spawn masses collected from the field, these embryos had been subjected to unknown conditions, and consequently of were of indeterminable age. It was therefore considered more reliable to assess embryonic development relative to a fixed developmental event such as hatching than to compare absolute ages. Accordingly, the developmental state of spawn masses from which experimental subjects were obtained was noted



as 'hatched', 'unhatched' and 'hatching'. *A. proxima* spawn masses may take several days to fully hatch; usually a few precocious hatchlings will be present on the first day, with the majority emerging during the subsequent day or two. Spawn masses denoted as 'hatching' were within the 3 d period following first hatching. Microscopic inspection confirmed that all embryos from unhatched spawn masses had attained late veliger stage.

#### Photomicroscopy

Photomicroscopy was used to illustrate the novel process of intracapsular metamorphosis in *A. proxima*. Embryos held within petri dishes were anaesthetized by the gradual addition of 15% magnesium chloride in TFSW. When the movement of the velar cilia ceased, specimens were transferred to a concave-welled microscope slide and photographs taken using a Leitz Diaplan microscope with a Wild Photoatomat MP545 attachment.

#### Experimental Protocol and Analysis

Embryos were divided into 3-5 replicates per treatment with 10 embryos and 10 veligers per replicate. Embryos and veligers were held in plastic 6-well flat bottomed polystyrene tissue culture plates (Corning Ltd., N.Y.) for Experiments 1-4, and in individual glass petri dishes (diameter 55mm, height 20mm) containing 15-20 ml of the treatment solution for the duration of all other experiments. Embryos used in Experiments 1-4 had been reared in a 6°C incubator (Gallenkamp) and so subsequent bioassays were run accordingly at 6°C. All other experiments were undertaken at ambient field temperature (10°C).

Sibling embryos were used within each separate experiment. Embryos were liberated from the stroma using a scalpel and fine forceps to allow the even partitioning of numbers between treatments and replicates. No visible morphological or behavioural differences between these liberated embryos (still intact within their capsules) and those embryos held within undisturbed stroma was observed.

Where possible hatched veligers, sibling to the embryos, were employed to act as positive controls. In the event that an experiment was initiated before the onset of natural hatching, the veligers were artificially hatched by rupturing the capsule wall with a fine tungsten needle (see Todd *et al.* 1991). Spawn mass size did not permit the use of sibling veligers in several experiments (Experiments 10 and 11) and it was necessary to utilise unrelated competent naturally hatched veligers of known age.

Each experiment was monitored using a Wild-E binocular microscope on Day +3 after the initiation of the experiment, and thereafter on Day +6 unless the positive control had previously elicited a maximal response or high mortality precluded it. Daily monitoring was undertaken only in Experiments 15 & 16, where the unknown nature of the cues necessitated the determination of rate of response in addition to absolute metamorphic response. In the absence of a suitable cue *A. proxima* veliger larvae may evacuate the larval shell. Such 'spontaneous evacuees' retain the morphology of the veliger larvae, and are therefore clearly distinct from those veligers casting their shells whilst undergoing metamorphosis. In the present study subjects were scored as pediveligers, spontaneous evacuees, successful metamorphs or dead.

The Minitab 8.1 Statistical Software Package was used for all data analysis within this study. Metamorphic responses were converted to proportions and thereafter normalized by angular transformation before analysis by one-way analysis of variance. Where a significant difference (at the  $P=0.05$  level) between treatment responses was established then multiple comparisons by Tukey's HSD test were performed on the arc-sine transformed data to identify the significant differences between treatment means. The resulting Tukey groupings consist of those metamorphic responses considered significantly different at the  $P<0.05$  level and are expressed within both Tables and Figures as superscript letters adjacent to the relevant responses. Data are expressed in all Tables and Figures as back-transformed means (presented in percentage form), and, where appropriate, are displayed with associated standard error bars.

### 3.3 RESULTS

#### The Effect of Choline Chloride on Embryos

Table 3.1 summarizes the results of 13 experiments designed to assess the comparative effects of both natural and artificial metamorphic cues on unhatched *A. proxima* embryos. The potential inductive capacity of each cue was confirmed by the employment of sibling veligers (with the exception of Experiments 10 & 11 in which non-siblings had to be used) as controls. The metamorphic responses of these hatched veligers are presented for comparison in Table 3.2.

Choline induced a significant level of metamorphosis (see Table 3.2 for back-transformed mean responses and relevant ANOVAs) in veligers employed as positive controls in every one of the 13 experiments. These results confirmed previous findings that choline is capable of the successful induction of metamorphosis in competent veligers of *A. proxima*. (Todd *et al.* 1991) Table 3.1 (column [B]) shows the percentage of intracapsular metamorphosis elicited amongst embryos in response to treatment with 5mM choline chloride. One-way analysis of variance performed upon the arc-sine transformed data (summarized in Table 3.1) confirmed significant levels of intracapsular metamorphosis were triggered by administration of choline in 12 of the 13 experiments. The comparative differences between the metamorphic responses of embryos and veligers are clearly illustrated by Fig 3.1. Experiment 2 represented the only occasion on which choline failed to trigger a significant level of intracapsular metamorphosis. Although 15.7% of embryos were induced to metamorphose, an unusually high between-replicate variation in metamorphic response may have precluded a significant result ( $F_{1,4}=4.0$ ,  $P=0.116$ ).

Mean intracapsular metamorphic responses, like those exhibited by veligers, showed appreciable variation between experiments (see Fig. 3.1). The maximum percentage of choline-induced intracapsular metamorphs (98.9%) was observed in Experiment 12. The minimum, although still significant ( $F_{2,1}=8.12$ ,  $P=0.006$ ), proportion of intracapsular metamorphs (11.3%) was produced in Experiment 15.

**Table 3.1. Final Metamorphic Responses of Embryos to Natural and Artificial Cues.** Data are back-transformed means for each quintuplicate treatment (except Experiments 1-4 and 9 which were triplicated) expressed as percentage responses. Letters in superscript denote Tukey groupings. Metamorphic responses in different Tukey groupings are considered different at the  $P=0.05$  level.

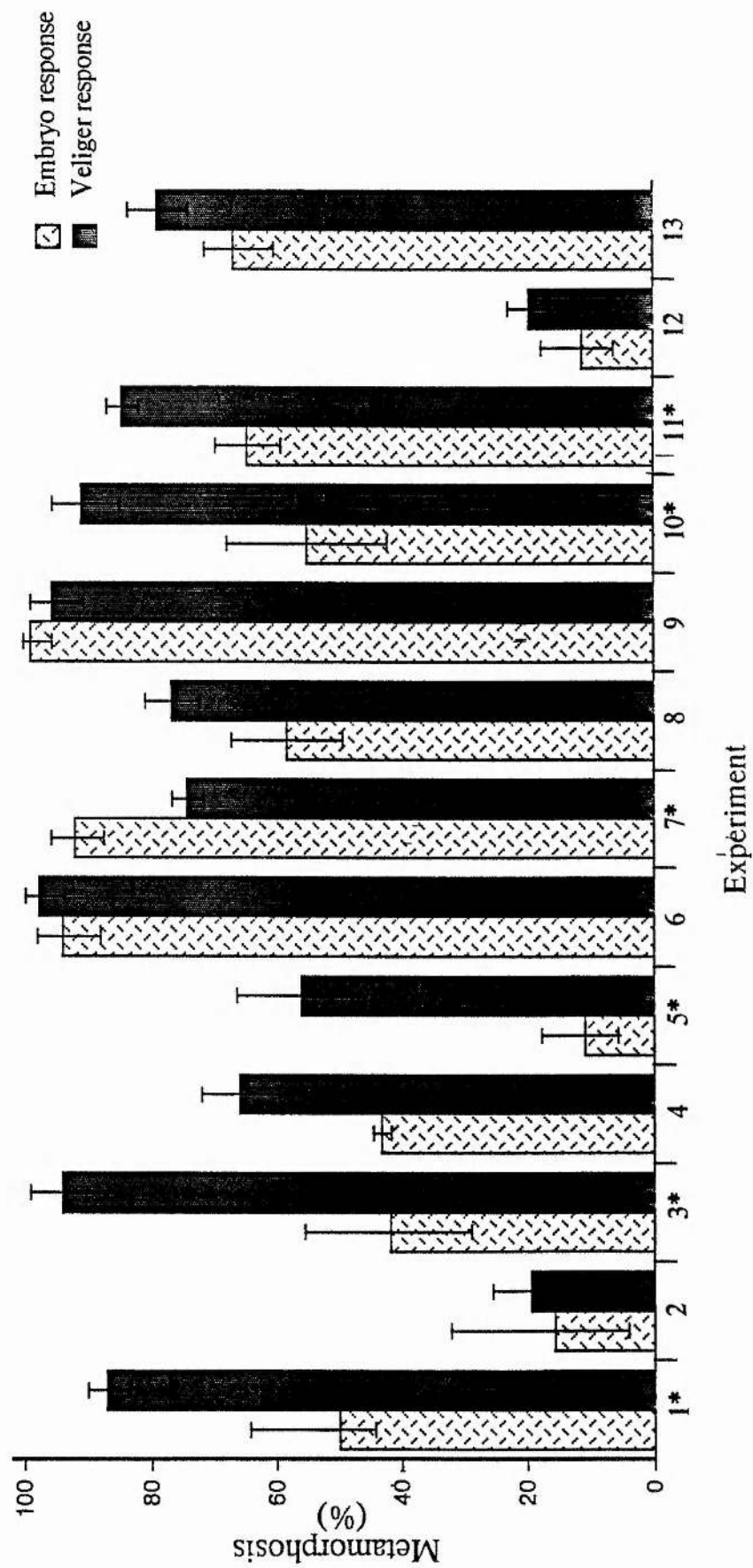
Expt	Treatment								ANOVA <i>F</i> value
	Sea water [A]	Choline [B]	19mM K <sup>+</sup> [C]	29mM K <sup>+</sup> [D]	CSW [E]	50% CSW [F]	20°C CSW [G]	Frozen CSW [H]	
1	0	50.0	-	-	-	-	-	-	182.5(1,4) P<0.001
2	0	15.7	-	-	-	-	-	-	4.0 (1,4) P=0.116
3	0	41.8	0	-	-	-	-	-	26.3(2,6) P=0.001
4	0	43.2	0	-	0	-	-	-	2247.5(3,8) P<0.001
5	0 <sup>a</sup>	10.9 <sup>b</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0.4 <sup>a</sup>	-	-	-	7.9 (4,20) P=0.001
6	0 <sup>a</sup>	93.9 <sup>b</sup>	0.4 <sup>a</sup>	-	-	-	-	-	109.4(2,12) P<0.001
7	0 <sup>a</sup>	92.0 <sup>c</sup>	64.5 <sup>b</sup>	-	-	-	-	-	149.0(2,12) P<0.001
8	0 <sup>a</sup>	58.3 <sup>b</sup>	-	-	6.1 <sup>a</sup>	-	-	-	31.3(2,12) P<0.001
9	0	98.9	-	-	0	-	0	0	186.3(4,10) P<0.001
10	0	55.1	-	-	0	0	-	-	41.22(3,16) P<0.001
11	0 <sup>a</sup>	64.5 <sup>b</sup>	-	-	0 <sup>a</sup>	0.4 <sup>a</sup>	-	-	122.5(3,16) P<0.001
12	0 <sup>a</sup>	11.3 <sup>b</sup>	-	-	0.4 <sup>a</sup>	-	-	-	8.1(2,12) P=0.006
13	0 <sup>a</sup>	66.6 <sup>b</sup>	0 <sup>a</sup>	-	0.4 <sup>a</sup>	-	-	-	104.5(3,16) P<0.001

**Table 3.2. Final Metamorphic Responses of Veligers to Natural and Artificial Cues.** Data are back-transformed means for each quintuplicate treatment (except Experiments 1-4 and 9 which were triplicated) expressed as percentage responses. Hatching status is shown as nat (naturally hatched veligers) and art (artificially hatched veligers). Letters in superscript denote Tukey groups. Metamorphic responses in different Tukey groupings are considered different at the  $P=0.05$  level. All veligers, except those used in Experiments 10 and 11, are siblings to each set of embryos presented in Table 3.1.

Expt	Status	Treatment								ANOVA
		Sea water [A]	Choline [B]	19mM K+ [C]	29mM K+ [D]	CSW [E]	50% CSW [F]	20°C CSW [G]	Frozen CSW [H]	
1	nat	1.1	87.0	-	-	-	-	-	-	87.2(1,4) P<0.001
2	art	0	19.4	-	-	-	-	-	-	37.2(1,4) P=0.004
3	nat	0 <sup>a</sup>	94.0 <sup>b</sup>	19.2 <sup>a</sup>	-	-	-	-	-	35.5(2,6) P<0.001
4	nat	7.23 <sup>a</sup>	65.8 <sup>c</sup>	53.3 <sup>c</sup>	-	19.8 <sup>b</sup>	-	-	-	56.2(3,8) P<0.001
5	nat	1.1 <sup>a</sup>	54.04 <sup>b</sup>	31.0 <sup>b</sup>	14.3 <sup>a/b</sup>	4.0 <sup>a</sup>	-	-	-	3.28(4,20) P=0.032
6	art	0 <sup>a</sup>	97.6 <sup>c</sup>	44.1 <sup>b</sup>	-	-	-	-	-	73.5(2,12) P<0.001
7	nat	0 <sup>a</sup>	74.2 <sup>c</sup>	58.2 <sup>b</sup>	-	-	-	-	-	281.9(2,12) P<0.001
8	nat	0 <sup>a</sup>	76.6 <sup>c</sup>	-	-	13.7 <sup>b</sup>	-	-	-	229.8(2,12) P<0.001
9	nat	0 <sup>a</sup>	95.5 <sup>b</sup>	-	-	13.0 <sup>a</sup>	-	1.1 <sup>a</sup>	1.1 <sup>a</sup>	42.4(4,10) P<0.001
10	nat	11.3 <sup>a</sup>	90.8 <sup>c</sup>	-	-	58.5 <sup>b</sup>	71.0 <sup>b/c</sup>	-	-	21.4(3,16) P<0.001
11	nat	0 <sup>a</sup>	84.4 <sup>c</sup>	-	-	37.8 <sup>b</sup>	35.7 <sup>b</sup>	-	-	159.8(3,16) P<0.001
12	art	0	19.6	-	-	0	-	-	-	125.6(2,12) P<0.001
13	art	0 <sup>a</sup>	78.7 <sup>c</sup>	28.7 <sup>b</sup>	-	19.6 <sup>b</sup>	-	-	-	137.7(3,16) P<0.001



**Figure 3.1. The metamorphic induction capacity of 5mM choline chloride on embryos and veligers.** Data are back-transformed means and associated standard errors (n=3 replicates per treatment in Experiments 1-4 and 9, and 5 replicates per treatment in all other experiments). With the exception of the embryonic response of Experiment 2, all choline induced metamorphic responses (both veliger and embryonic) are considered significant relative to the sea water control ( $P<0.05$ ). Asterisks denote those experiments in which a significant difference between the mean metamorphic response of embryos and veligers ( $P<0.05$ ) was established.



### Differences in Response to Choline by Naturally and Artificially Hatched Veligers

Veligers used to act as positive controls were supplied by artificial hatching when the spawn mass in question had not yet begun to hatch naturally. The hatching status, either artificial or natural, of veligers for each experiment is displayed in Table 3.2 with accompanying metamorphic responses. The possibility that artificially hatched veligers subjected to choline might respond differently to naturally hatched veligers as a consequence of being morphologically or physiologically 'premature' was addressed by a comparison of means. No significant difference in level of response between artificially hatched veligers (mean 57.0%,  $n=4$ ) and naturally hatched veligers (mean 82.4%,  $n=9$ ) was established ( $F_{1,11}=2.54$ ,  $P=0.139$ ).

### Embryonic Age and Response to Choline

The relationship between relative age of embryos and the induction of intracapsular metamorphosis was investigated by comparing the metamorphic responses of embryos from spawn masses categorised as 'hatched', 'hatching', or 'unhatched'. Table 3.3 presents the back-transformed mean metamorphic responses of embryos to choline for each developmental state and illustrates the appreciable variation in intracapsular metamorphic response within 'developmental state' categories. Nevertheless, the grand mean levels of intracapsular metamorphosis in embryos from unhatched, hatching and hatched spawn masses appear little different at 51.18%, 51.45% and 58.44% respectively. A one-way analysis of variance established no significant difference between these classes at the  $P<0.05$  significance level ( $F_{2,10}=0.11$ ,  $P=0.899$ ).

### Description of the Metamorphic Process in Response to Choline

The process of intracapsular metamorphosis appeared to proceed identically to the normal process of extracapsular metamorphosis exhibited by *A. proxima* larvae in response to choline chloride. No morphological or behavioural anomalies in the metamorphic process itself were visibly apparent (see Plate 3.1a). On evacuation of the larval shell, however, newly metamorphosed juveniles enclosed

**Table 3.3. Metamorphic responses of embryos from unhatched, hatching and hatched spawn masses subjected to 5mM choline.** Statistical significance of the embryonic metamorphic response to choline is denoted by \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Absolute age of embryos collected from the field could not be determined and so developmental state was judged relative to the hatching of the rest of the spawn mass. ANOVA performed upon the arc-sine data revealed there to be no significant differences between the grand mean metamorphic responses of each developmental state.

Experiment	Site of Origin	Developmental status of spawn mass at initiation of experiment		
		Unhatched	Hatching	Hatched
1	Kinkell Braes	-	-	50.0***
2	Cuan Ferry	15.7	-	-
3	Cuan Ferry	-	-	41.8***
4	Kingsbarns	-	-	43.2***
5	Kingsbarns	-	10.9***	-
6	Clachan Seil	93.9***	-	-
7	Clachan Seil	-	92.0***	-
8	Clachan Seil	-	-	58.3***
9	Clachan Seil	-	- -	98.9***
10	Clachan Seil	55.1***	-	-
11	Clachan Seil	64.5***	-	-
12	Clachan Seil	11.3*	-	-
13	Clachan Seil	66.6***	-	-
Grand Means		51.18	51.45	58.44

within the egg capsule commenced crawling upon the exterior shell surface. The subsequent decrease in available volume caused by the presence of both metamorph and larval shell within the egg capsule resulted in visible deformation of the capsule membrane (see Plate 3.1b). Artificial rupturing of the capsule wall using a fine tungsten needle and forceps resulted in the liberation of metamorphs morphologically indistinguishable from those of naturally hatched veliger origin (see Plate 3.2). Whilst unhatched metamorphs were cultured for several days post-induction of metamorphosis, none were observed to hatch.

### The Effect of Elevated Potassium on Embryos

The mean metamorphic responses displayed by sibling veligers in response to 19 mM potassium are detailed in Table 3.2 (column [C]) with relevant ANOVAs. Potassium was assayed in a total of six experiments, and caused a significant level of metamorphosis in five of these.

It is apparent from Table 3.1, in which mean levels of intracapsular metamorphosis in response to 19mM potassium are displayed (column [C]) for each of the 6 relevant experiments, that excess potassium was unsuccessful in triggering intracapsular metamorphosis. Thus, unlike choline, potassium appears to elicit differing in responses between embryos and hatched veligers. The contrasting levels of embryo and veliger potassium-mediated metamorphosis are illustrated in Fig.3.2.

An exception to the trend was provided by Experiment 7. With a mean response of 64.5%, it was the only experiment in which a significant level of intracapsular metamorphosis was induced by potassium. The cause of this atypical result is unknown and whilst it may be attributable to contamination by choline, a subsequent Tukey's HSD test showed the response to be significantly lower than that induced by the choline treatment (see Table 3.1, column [B]).

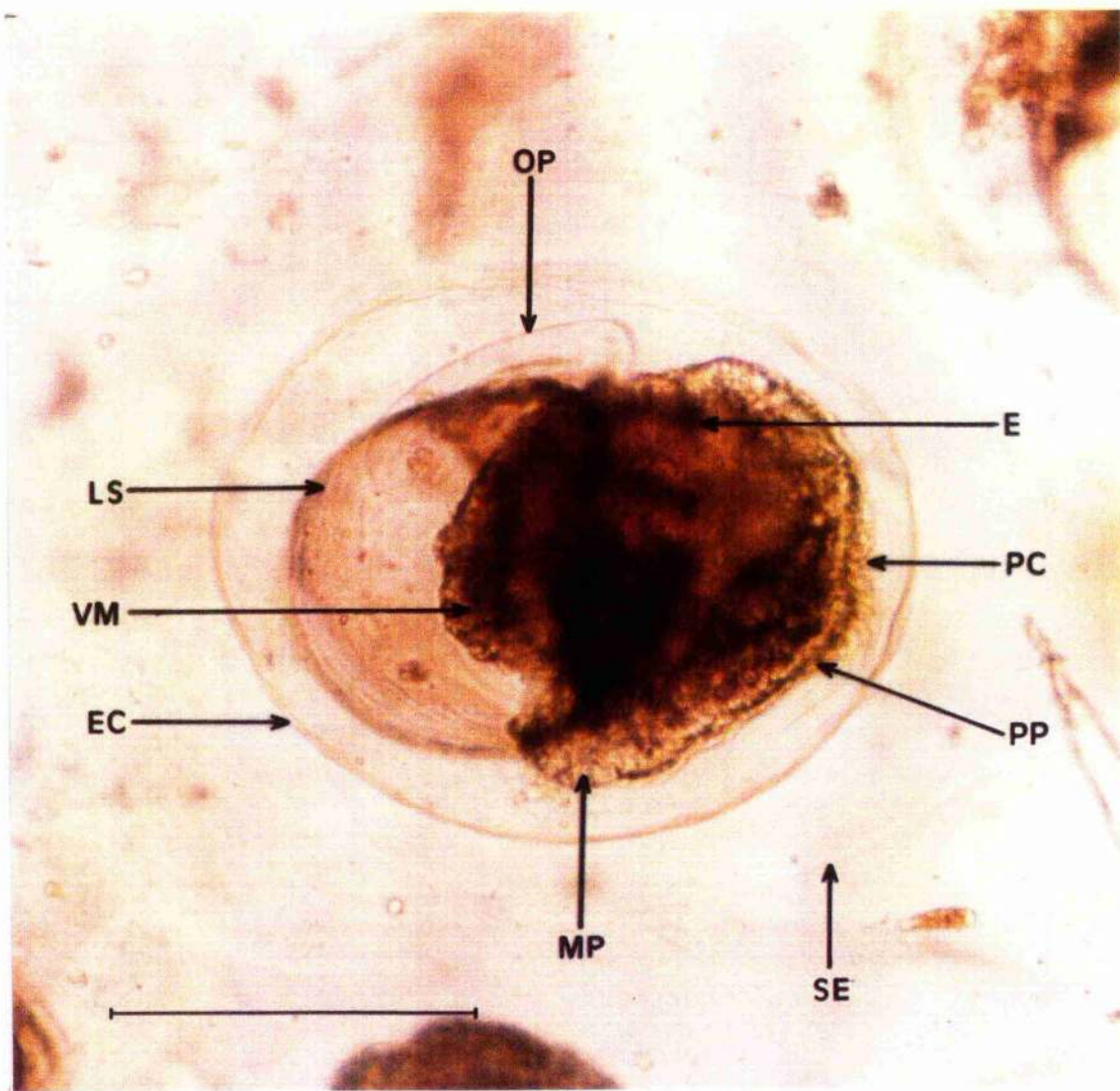
Whilst these embryos failed to metamorphose in response to elevated potassium ion concentration, sibling embryos from each experiment were shown to be competent in response to choline (see Table 3.1 column [B]). The failure of potassium to induce intracapsular metamorphosis relative to the success of choline chloride was unexpected and the cause unknown. The lack of response may have been attributable to precompetence of the embryos with respect to potassium or to

**Plate 3.1a. Photomicrograph of an intracapsular metamorph; right latero-dorsal aspect.** The larval shell (LS) and operculum (OP) have been shed and the velum completely resorbed whilst the metamorph is still within the egg capsule. The visceral mass (VM) and metapodium (MP) can be seen to have not yet fully fused. Scale bar represents 200µm.

Additional abbreviations used in plate show:

- EC    egg capsule (primary egg membrane)
- SE    secondary egg membrane
- PP    propodium
- PC    propodial cilia
- E     eye





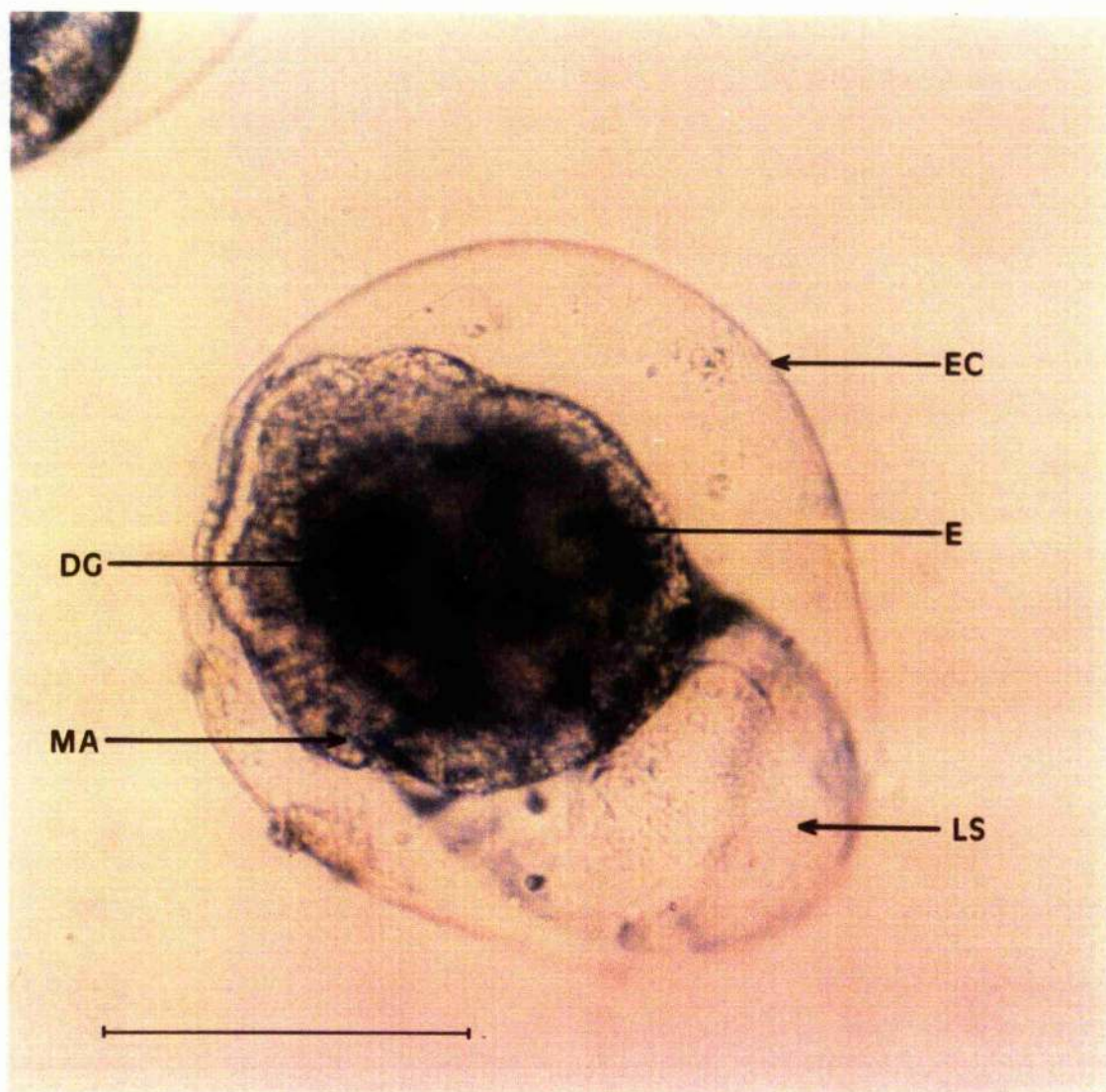
**Plate 3.1b. Photomicrograph of an intracapsular metamorph; dorsal aspect.** Deformation of the egg capsule membrane (EC) resulting from both the evacuated larval shell (LS) and metamorph remaining confined within the egg capsule is clearly visible. Scale bar represents 200 $\mu$ m.

Additional abbreviations used in plate show:

DG digestive gland

MA mantle

E eye



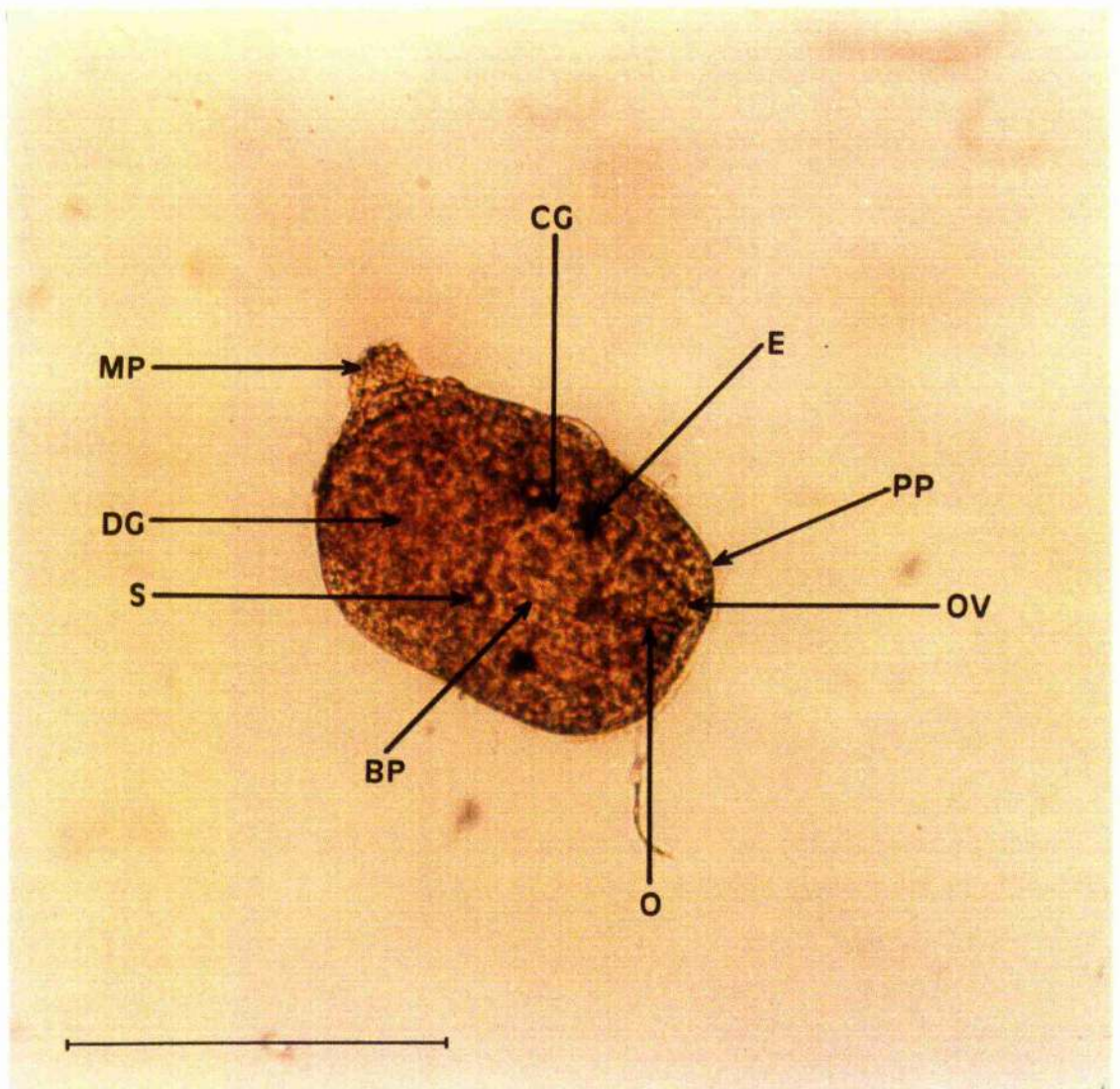
**Plate 3.2. Photomicrograph of a benthic juvenile: dorsal aspect.**

Complete metamorphosis is indicated by the shedding of shell and operculum, resorption of velar lobes and formation of the oral veil (OV), detorsion, the anterior position of buccal pump (BP) relative to the cerebral ganglion (CG) and flattening of the body form. It is apparent that the juvenile product of intracapsular metamorphosis does not appear morphologically distinct from that of normal larval metamorphosis. Scale bar represents 200 $\mu$ m.

Additional abbreviations used in plate show:

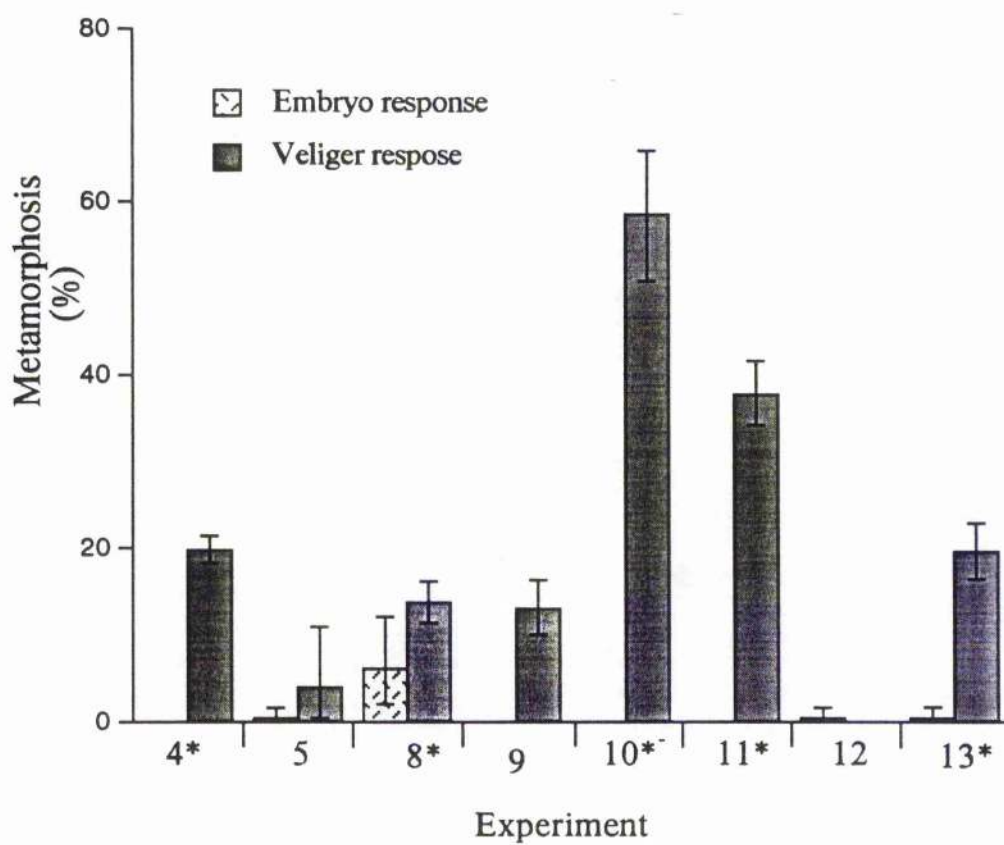
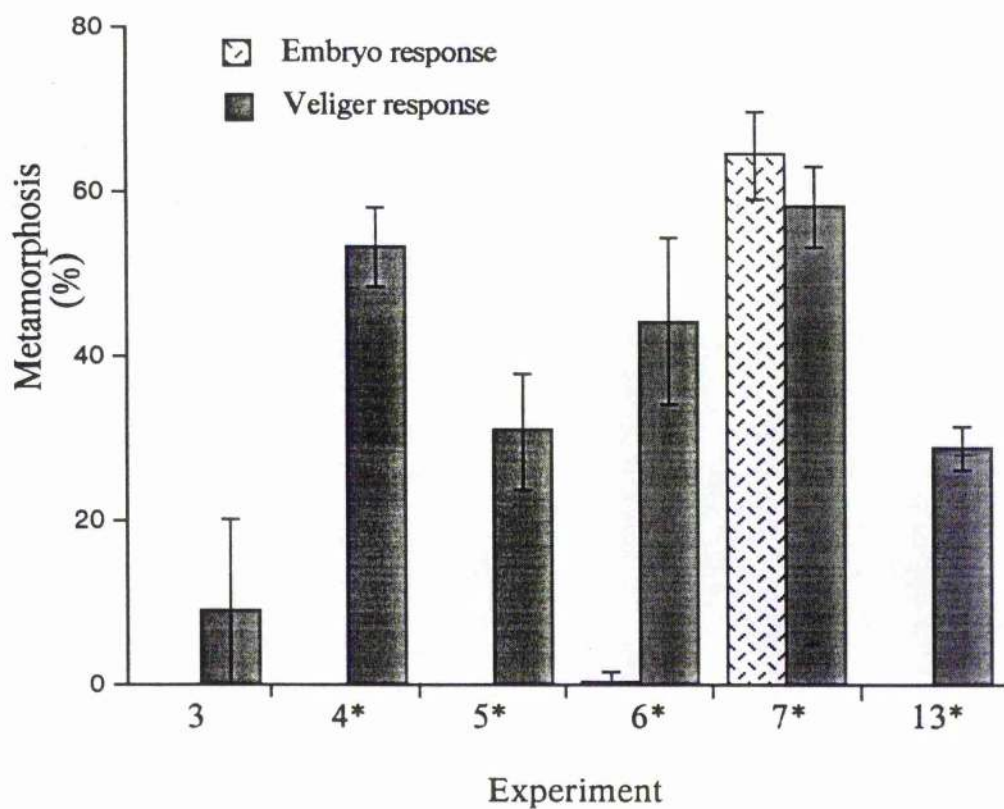
MP	metapodium
DG	digestive gland
S	statocyst
O	oesophagus
PP	propodium
E	eye





**Figure 3.2 (above).** The metamorphic induction capacity of 19mM potassium on embryos and veligers. Data are back-transformed means and associated standard errors (n=3 replicates per treatment in Experiments 3 and 4, and 5 replicates per treatment in all other experiments). Asterisks denote those experiments in which excess potassium elicited a significant veliger response at the  $P<0.05$  level. With the exception of Experiment 7, embryos proved insensitive to 19mM potassium.

**Figure 3.3 (below).** The metamorphic induction capacity of *Electra pilosa* conditioned sea water (CSW) on embryos and veligers. Data are back-transformed means and associated standard errors (n=3 replicates per treatment in Experiments 4 and 9, and 5 replicates per treatment in all other experiments). Asterisks denote those experiments in which CSW elicited a significant veliger response at the  $P<0.05$  level and was therefore considered 'active'. In no experiments did embryos demonstrate a significant metamorphic response towards CSW concurrently proven to be 'active'.





an insufficient effective potassium ion concentration reaching the embryos. To compensate for any possible reduction in the effective dose of potassium, and thereby to ensure the administration of a sufficient effective dose of potassium to the embryo, an extra treatment of 29mM potassium was introduced (Experiment 5). Tukey's HSD for Experiment 5 established that whilst the 19mM treatment elicited a significant 31.0% of veligers (sibling to the embryos) to metamorphose, 29mM potassium triggered only a marginal veliger response of 14.3% metamorphosis (see Table 3.2, Column [D]). Neither the 19 mM or 29mM potassium treatments had any inductive effect on embryos (see Table 3.1, columns [C] and [D]).

#### Differences in Response to Potassium by Naturally and Artificially Hatched Veligers

The response to potassium by naturally and artificially hatched veligers was investigated using an identical rationale to that employed in the bioassay of choline. A one-way ANOVA on the arc-sine transformed metamorphic responses revealed no significant difference between naturally hatched (mean 40.1%,  $n=4$ ) and artificially hatched (mean 36.2%,  $n=2$ ) larvae ( $F_{1,4}=0.07$ ,  $P=0.806$ ).

#### The Effects of Conditioned Sea Water (CSW) on Embryos

The inductive properties of *Electra pilosa* conditioned sea water (CSW) were investigated in a series of eight experiments using embryos and sibling veligers. CSW elicited significant levels of extra-capsular metamorphosis in five of the eight experiments (see Table 3.2, column [E]).

Of those five experiments in which CSW was shown to be "active", that is, capable of inducing significant levels of extracapsular metamorphosis (Experiments 4, 8, 10, 11 & 13), not one elicited a significant level of metamorphosis among sibling embryos (see Table 3.1, column [E]). Whilst these embryos failed to respond to CSW, in each experiment sibling embryos were demonstrated to be competent to choline chloride. The comparative responses of embryos and veligers subjected to CSW are illustrated for all eight experiments in Fig. 3.3. It can be seen that although there is clear variability in the mean metamorphic response of veligers, there is a consistent absence of any significant embryonic metamorphic induction.

Experiments performed during the characterisation of the natural cue have indicated the presence of a dilution effect in the action of the cue (see Chapter 4). Bioassay of solutions of CSW ranging from 100% down to 0.0001% of the original concentration revealed a maximum inductive effect between 50% and 100% CSW. It therefore appeared logical, given the lack of successful intracapsular metamorphosis induced by undiluted CSW, that 50% CSW treatments be included in Experiments 10 & 11. Whilst significant levels of extracapsular metamorphosis were induced (see Table 3.2, column [F]) by 50% CSW, Tukey's HSD for each experiment showed that these outcomes could be considered no higher than the responses elicited in veligers by undiluted CSW (column [E]). Moreover, no embryos were induced to metamorphose. Within this series of experiments, therefore, it is apparent that diluted CSW had, like undiluted CSW, no capacity to induce intracapsular metamorphosis (see Table 1) whilst functioning as an effective induction agent for hatched veliger larvae.

#### Differences in Response to CSW by Naturally and Artificially Hatched Veligers

The provision of artificially hatched veligers was necessary only when no naturally hatched larvae were available. Whilst investigating the inductive properties of the natural metamorphic cue this situation arose only once, in Experiment 13. Whilst statistical analysis was precluded by the sample size, within the context of observed inter-experiment variability in metamorphic responses, no clear difference between naturally (mean 32.3%, n=4) and artificially hatched veligers (mean 19.6%, n= 1) subjected to active CSW was apparent.

#### Comparison of All Three Metamorphic Cues

In compiling the 'grand mean' responses the objective was to demonstrate comparative differences in sensitivity towards cues by unhatched and hatched larvae. This was facilitated by the inclusion only of those experiments in which the relevant cue had been demonstrated to be capable of eliciting a significant metamorphic response amongst competent hatched veliger larvae. For example, although Tables 3.1 and 3.2 show that six experiments using 19mM potassium were run, the grand mean responses for embryos and veligers consist of only those treatments in which potassium was confirmed to be active upon veligers, resulting

in the exclusion of Experiment 3 from the data group (see Table 3.4). The resultant back-transformed grand mean metamorphic responses of both veligers and embryos to all cues are displayed in Table 3.4.

It is clear that hatched veligers underwent significant levels of metamorphosis on exposure to both natural (*Electra pilosa* conditioned sea water) and artificial (5mM choline and 19mM potassium) cue treatments ( $F_{3,32}=43.2$ ,  $P<0.001$ ). Subsequent multiple comparison by Tukey's HSD established that choline chloride elicited a significantly greater response (mean 75.3%) than all other cues. Whilst both potassium and CSW (means 42.9% and 28.9% respectively) induced significant levels of extracapsular metamorphosis, the levels of response were statistically indistinguishable from each other.

In contrast to the response of the veligers, embryos showed no appreciable metamorphic response to either 19mM potassium (mean 3.9%) or *Electra pilosa* conditioned sea water (mean 0.4%). Choline chloride did, however, cause metamorphosis in a mean 55.9% of embryos, and was therefore the only cue found to elicit a significant level of intracapsular metamorphic response ( $F_{3,32}=24.67$ ,  $P<0.001$ ). Fig. 3.4. shows the back transformed mean levels of metamorphosis of embryos and veligers subjected to natural and artificial cues in graphical format and clearly illustrates the disparity in response between embryos and veligers towards the same cues.

Although lower than the mean value of choline-induced metamorphosis established for hatched veligers (55.9% compared to 75.3%), there was no actual statistical difference ( $DF=23$ ,  $P=0.13$ ) between the mean responses of embryos and veligers toward 5mM choline.

#### Other Neuroactive Compounds

The results of experiments in which additional neuroactive agents were screened for morphogenic activity are displayed in Table 3.5. Both embryos and veliger larvae proved insensitive to all L-DOPA ( $10^{-4}M$ ) and dopamine ( $10^{-2}$  to  $10^{-5}M$ ) treatments.

Whilst GABA had no morphogenic effect on embryos, veliger larvae did show a weak response. Whilst 5mM choline produced a significant response by

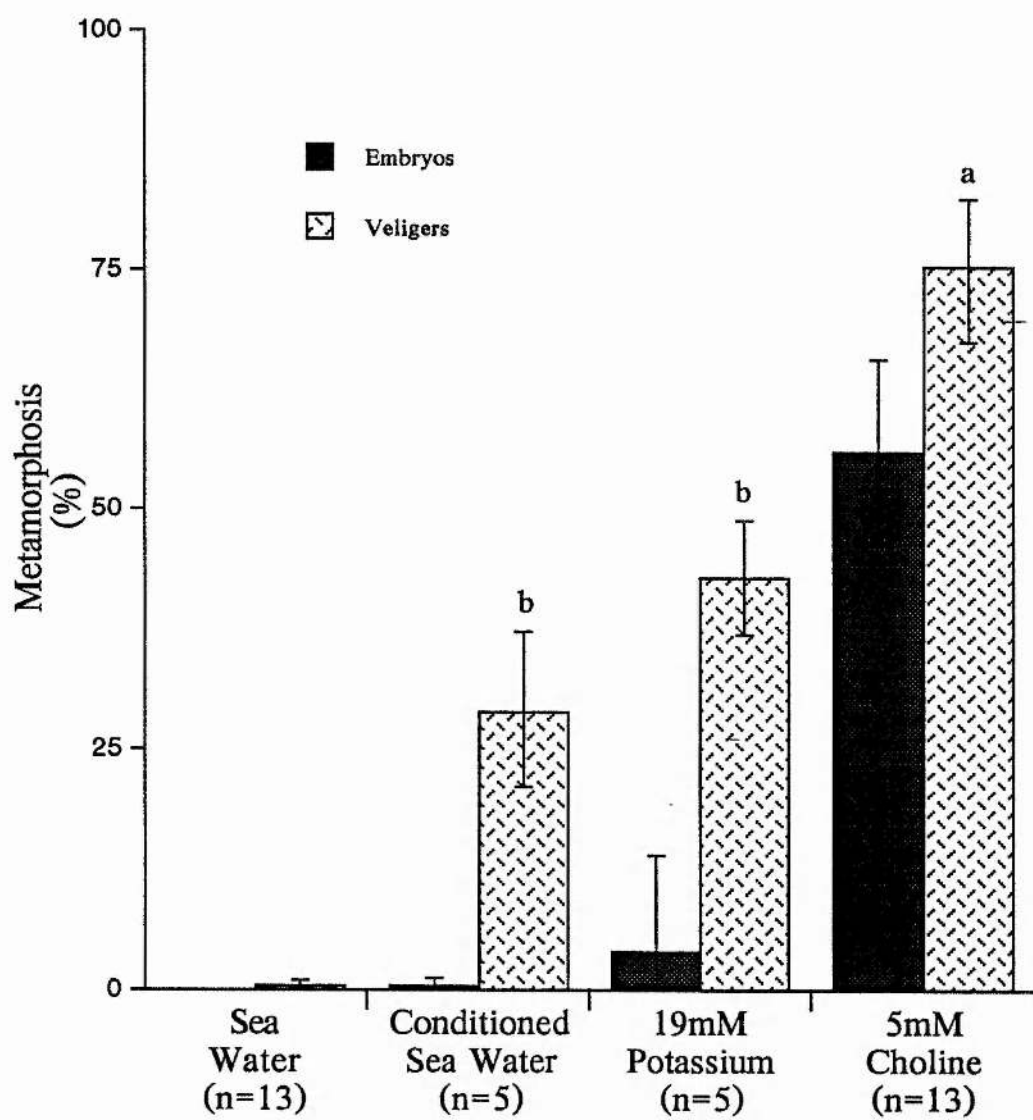
**Table 3.4.**(above right) Back-transformed mean metamorphic responses for embryos and veligers subjected to artificial (5mM choline chloride and 19mM potassium) and natural (Conditioned Sea Water) metamorphic cues. Superscript type indicates groupings resulting from Tukeys' HSD performed separately on the responses of the embryos and veligers. Separate Tukey groups are considered different at the  $P=0.05$  significance level. Integers in parentheses denote the number of experiments composing the mean. These mean metamorphic responses are portrayed graphically in Fig. 3.4.

**Table 3.5.**(below right). Back-transformed mean metamorphic responses of veligers subjected to other neuroactive agents (L-DOPA, GABA and dopamine). Asterisks denote those responses considered significantly different to the sea water controls at the  $P<0.05$  level.

Treatment	Back-transformed Mean Metamorphic Response (%)	
	Embryos	Veligers
Sea Water Control	0 <sup>a</sup> (13)	0.4 <sup>A</sup> (13)
5mM Choline	55.9 <sup>b</sup> (13)	75.3 <sup>C</sup> (13)
19mM Potassium	3.9 <sup>a</sup> (5)	42.9 <sup>B</sup> (5)
Conditioned Sea Water	0.4 <sup>a</sup> (5)	28.9 <sup>B</sup> (5)

Treatment		Experiment		
		14	15	16
Sea water control		3.7	0	0
Choline control		52.4*	95.5*	83.3*
L-DOPA	10 <sup>-4</sup> M	6.7	-	-
GABA	10 <sup>-2</sup> M	-	2.4	-
	10 <sup>-3</sup> M	-	23.2*	-
	10 <sup>-4</sup> M	-	4.5	-
	10 <sup>-5</sup> M	-	0	-
	10 <sup>-6</sup> M	-	0	-
	10 <sup>-7</sup> M	-	0	-
Dopamine	10 <sup>-2</sup> M	-	-	0
	10 <sup>-3</sup> M	-	-	0
	10 <sup>-4</sup> M	-	-	0
	10 <sup>-5</sup> M	-	-	0

**Figure 3.4.** Back-transformed grand mean responses of embryos and veligers subjected to artificial (19mM potassium and 5mM choline) and natural (*E.pilosa* conditioned sea water) metamorphic cues. Multiple comparisons were performed on the veliger responses by Tukeys' HSD and the resultant Tukey groupings considered significantly different at the  $P < 0.05$  level are displayed above the relevant columns. Figures in parentheses indicate the number of experiments which compose the mean. The corresponding absolute values of these back-transformed grand mean responses are displayed in Table 3.4.

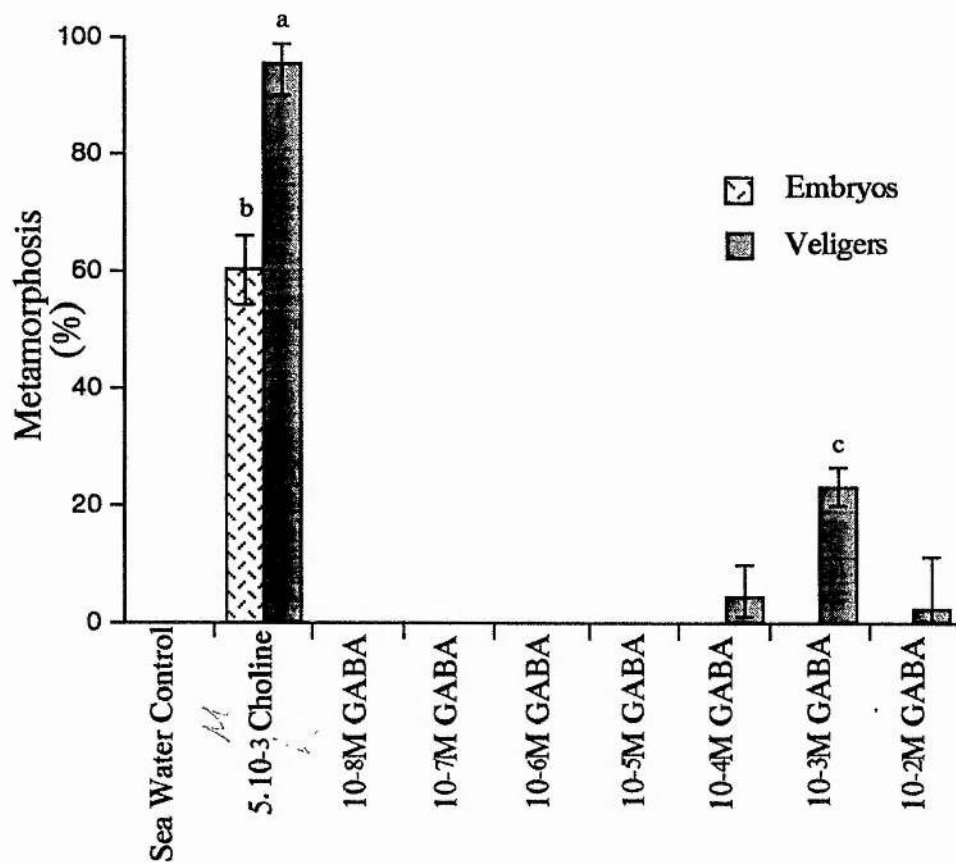
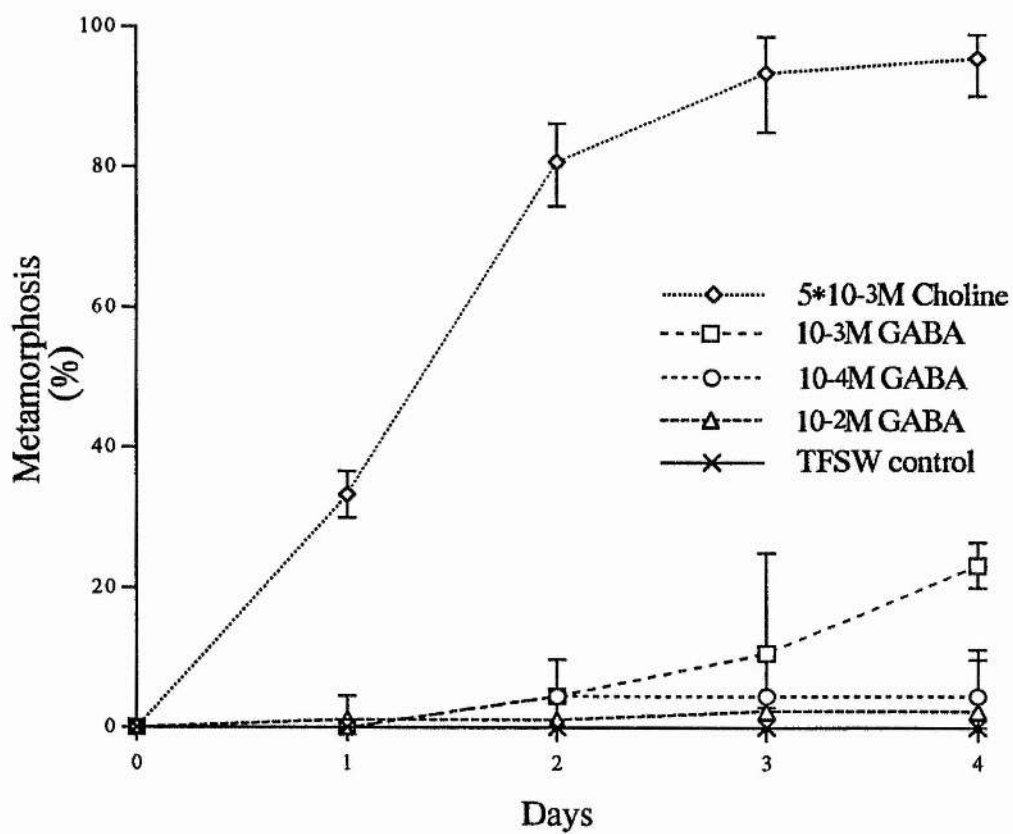




day +1 and a maximal response of 95.5% on day+4, the optimum dose of GABA produced a significant response only on the final day. Although a range of  $10^{-2}$ - $10^{-7}$ M GABA was used, the sole active treatment was found to be  $10^{-3}$ M, which triggered a significant (albeit low) response of 23.2% (see Figs. 3.5 a ). Multiple comparison by Tukey's HSD revealed that whilst the final metamorphic response elicited by GABA was greater than the TFSW negative control, it was significantly lower than that of choline (see Fig.3.5b).

**Figure 3.5a (above right).** Artificially induced (5mM choline and GABA) metamorphic responses of hatched veliger larvae. Data are back-transformed daily mean metamorphic responses and associated standard errors. Whilst a range of  $10^{-2}$ - $10^{-8}$ M GABA was screened, only those concentrations which elicited a response ( $10^{-2}$ - $10^{-4}$ M) are shown.

**Figure 3.5b (below right).** Final metamorphic responses of embryos and hatched veliger larvae to  $10^{-2}$ - $10^{-8}$ M GABA and 5mM choline control. Data are back-transformed cumulative mean metamorphic responses and associated standard errors. Multiple comparison of the veliger responses performed by Tukeys HSD established only the choline control and  $10^{-3}$ M GABA treatments to have elicited significant levels of metamorphosis. The resulting groupings, considered significantly different at the  $P < 0.05$  level are displayed above the relevant responses.



### 3.4 DISCUSSION

#### Morphogenic Properties of Choline

The well established presence of a precompetent period in *A. proxima* veliger larvae of 24-48 hours (Thompson, 1958a, Todd *et al.*, 1991) has previously precluded consideration that metamorphosis may be induced prior to hatching. The results of this investigation demonstrate that metamorphosis can indeed be induced in *A. proxima* before eclosion. This study constitutes the first occasion on which intracapsular metamorphosis has been reported for this species.

Poecilogony, that is, variation in the mode of development displayed within a species, represents an extreme case of variation in developmental state (and consequently attainment of competence) between progeny. Poecilogonous development within a species may be seasonal, geographical between populations, or mediated by environmental conditions (Hoagland & Robertson, 1988). First propounded by Giard over a hundred years ago and expanded on by him in, 1905, there have been many reported cases of poecilogony among marine invertebrates in the literature, few of which have withstood later critical evaluation (see reviews of Bonar, 1978; Hoagland & Robertson, 1988; Bouchet, 1989). For example, from a total of 42 species of marine mollusc reported to exhibit some degree of poecilogony, Hoagland & Robertson (1988) supported the status of only one, *Elysia chlorotica* (Family Elysiidae). Adults taken from allopatric populations displayed different reproductive extremes, one population producing planktotrophic veligers, the other producing crawl-away juveniles. Interbreeding of these produced offspring with hybrid reproductive characteristics (West *et al.*, 1984). In a systematic examination of literature alluding to poecilogony within the Class Gastropoda, Bouchet (1989) defined poecilogony as only that intraspecific variation in reproductive mode involving both planktotrophic and non-planktotrophic morphs, thus classing lecithotrophic pelagic veligers and crawl-away juveniles together. Under these terms, he concluded that convincing evidence in support of poecilogony was absent among the prosobranchia and shelled opisthobranchs, yet was displayed by certain members of the Ascoglossa and perhaps the Nudibranchia.

Interbreeding adults of the eolid opisthobranch *Tenellia pallida* from discrete populations were found by Eyster (1979) to produce one of two sizes of egg, 70µm diameter zygotes producing pelagic veliger larvae, and 100µm eggs resulting in veligers which metamorphosed prior to hatching, (although the shell was not shed until after hatching). Whether this is an example of true geographical poecilogony is disputable, since veliger larvae were not cultured post-hatching, leaving their developmental type (pelagic lecithotrophic or planktotrophic) open to question.

Considerable variation in the length of the precompetent period, resulting from the employment of disparate developmental modes, has since been observed in the cephalaspidean *Haminaea callidegenita* (Gibson & Chia). Approximately 50 to 70% of progeny hatch as crawl-away juveniles whilst the remaining embryos emerge as free swimming lecithotrophic veligers (Gibson & Chia, 1989a, 1991), undergoing a pelagic phase of up to 20 days (Gibson & Chia, 1991), before being induced to metamorphose in response to cues associated with a suitable juvenile habitat (Gibson, 1993). The relative proportion of each development mode present in a given spawn mass is flexible (Gibson & Chia, 1991) and is influenced by the presence of egg mass jelly (stroma) (Gibson & Chia, 1989a) and the developmental point (prior or post-hatching) at which competence is acquired (Gibson, 1993; Gibson & Chia, 1994). Crawl away juveniles, therefore, were the result of precocious attainment of competence whilst still within the primary capsule and consequent intracapsular metamorphosis triggered exclusively (Gibson & Chia, 1989a) by the surrounding egg mass jelly. Whilst *H. callidegenita* obviously displays intraspecific variation in mode of development and is cited by the authors as capable of poecilogonous development, only non-pelagic progeny have been documented. Consequently, this does not constitute poecilogony as defined by Bouchet (1989).

It is postulated that poecilogony may confer the advantages of direct recruitment of crawl-away juveniles to the population, where the adult food source may be exploited immediately, whilst pelagic veligers ensure that dispersal ability is retained (Eyster, 1979; Gibson & Chia, 1989a, Carroll & Kempf, 1990; Gibson & Chia, 1994).

Much of the confusion surrounding previously reported cases of poecilogonous development by gastropods may be attributed to the misidentification of morphologically similar species (see Hoagland & Robertson, 1988; Bouchet,

1989; and references therein). For example, a reported case of seasonal poecilogony observed in the opisthobranch *Elysia cauze* displaying spring planktotrophic, summer lecithotrophic, and winter direct developing developmental modes (Clarke *et al.* 1979) was later attributed to the sequential reproduction of several species (Jensen & Clarke, 1983). An apparent case of the gastropod *Crepidula dilatata* producing both direct developing and pelagic larvae was, on closer examination of adult morphology and reproductive behaviour, established to be the different reproductive modes of two separate, yet morphologically similar, species (Gallardo, 1979). Some examples may, in fact, display intraspecific variation within a reproductive mode i.e. non-pelagic, what Hoagland & Robertson (1988) termed 'incipient poecilogony'. That is, the employment of lecithotrophic veligers with a minimal precompetent phase and apparently plastic hatching point, such that hatching may occur just before, or just after, metamorphosis (Bouchet, 1989).

An example of exogenously (environmentally) mediated poecilogony is documented by Carroll & Kempf (1990). Spawn masses of the nudibranch *Berghia verrucicornis* deprived of aeration during culture, produced not lecithotrophic veligers but instead varying proportions of embryos underwent intracapsular metamorphosis and subsequently hatched as crawl-away juveniles. The absence of any difference in development time to hatching between aerated and unaerated cultures would imply that limitation in gaseous exchange was not a valid explanation. Indeed, the authors attributed the switch in developmental mode to agitation of the spawn mass during the process of aeration, acting by affecting the intrinsic mechanisms responsible for the onset of competence and metamorphosis, or the actual process of metamorphosis.

Variability in the age at attainment of competence has been reported previously for *Adalaria proxima* (Todd *et al.*, 1991), indeed, occasional instances of almost immediate post-hatching metamorphosis have been observed (Todd *et al.*, 1991). The differential attainment of competence displayed by *A. proxima*, such as that observed by Todd *et al.* (1991), may be attributable to either asynchronous development within the spawn mass resulting in the precocious attainment of competence by a few larvae, or to the physical impedance of eclosed larvae from hatching resulting in a proportion of veligers completing precompetent period before hatching (reviewed by Havenhand, 1991). The successful induction of

metamorphosis before hatching demonstrated within this study is not thought to be a consequence of either phenomenon.

Asynchrony in spawn mass development due to restricted gaseous exchange may be exacerbated by still, unaerated culture conditions. The rate of gaseous diffusion is proportional to the diffusion gradient, and the maximal rate of diffusion requires a high diffusion gradient. A flow of fresh water over the surface of the spawn mass will maintain a high diffusion gradient whilst still water will allow it to decline. Spawn masses were cultured without aeration during this study, but daily water changes resulted in considerable agitation of each spawn mass. In addition, regular microscopic inspection of spawn masses for determination of developmental status failed to reveal any differential development between embryos. Moreover, the metamorphosis of a few precocious individuals resulting from asynchronous development is not considered consistent with the high and repeatable levels of metamorphosis induced within this study. Therefore although it cannot be categorically discounted, there seems little compelling evidence in support of asynchrony in developmental rate within spawn masses as an explanation for differential attainment of competence in *A. proxima*. Any variation in hatching time, or apparent variation in the attainment of competence, may more likely be attributed to mechanical impairment of eclosed larvae from hatching by the retarded breakdown of the spawn mass membrane. The observations of eclosed *A. proxima* veliger larvae crawling captive within the outer spawn mass membrane by Havenhand & Todd (unpub. obs.) in Havenhand (1991) would tend to concur with this hypothesis. For the purposes of this study 'embryos' were classed as progeny which had not yet undergone eclosion and were therefore still within the primary egg membrane, precluding any possible influence of this source of variation in the attainment of competence on this investigation.

The diverse origins and histories of successful choline-induced intracapsular metamorphs would imply the phenomenon of intracapsular metamorphosis to be not merely specific to one population, or to a particular set of (sub-optimal) culture conditions, but rather a repeatable process which can be reliably induced under a predictable combination of conditions. Table 3.3 illustrates the sources of the different populations from which embryos were successfully induced to undergo intracapsular metamorphosis. In addition to the Scottish populations cited (Kinkell Braes and Kingsbarns, Fife, Cuan Ferry and Clachan Seil, Argyll) embryos in successful preliminary experiments included those from



Portaferry, N.Ireland and Menai Bridge, Anglesey. Successful intracapsular metamorphs also included those spawned and cultured wholly within the laboratory (Experiment 1-4), those collected from the field (Exps 5-13) and those incubated at either 6°C or 10°C.

The initiation of metamorphosis of hatched veliger larvae on continuous exposure to 5mM choline chloride concurs with the findings of Todd *et al.* (1991) in which continuous exposure of competent hatched veligers to an optimum dose of 5-10mM choline chloride induced maximal levels of metamorphosis in *A. proxima*. It is noteworthy that 5mM choline is apparently as equally effective in triggering intracapsular (mean 55.9%, n=13) as extracapsular (mean 75.3%, n=13) metamorphosis. This would imply that the biochemical steps in the pathway(s) active in choline-mediated initiation of metamorphosis unexpectedly become functional at some point prior to hatching in this species.

The mechanism by which choline and associated compounds act on invertebrate larvae to initiate metamorphosis has been the focus of several studies. Much information concerning the nature of metamorphic mechanisms has been inferred from comparative exposure times, effective dose concentrations and comparisons of latency periods displayed on exposure to both natural and artificial cues. Hadfield (1978a) compared the nature of morphogenetic activity in a series of 15 choline associated compounds and the natural cue for the tropical nudibranch *P. sibogae*. The nature of choline action apparently differed from that of the natural inducer by exhibiting a latency period of 3-4 days, whilst naturally induced metamorphosis required just 1 day. *P. sibogae* has been used as a model experimental system in many subsequent studies concerning the induction and physiology of metamorphosis. In a comparison of the natural metamorphic inducer and choline chloride, Hirata & Hadfield (1986) exposed precompetent and competent larvae to combinations of inducers in an attempt to determine the relative sites of action. The habituation displayed by precompetent larvae in response to the natural inducer was not evident in response to premature exposure to choline. Moreover, larvae of *P. sibogae* habituated to the natural inducer were capable of choline-induced metamorphosis, and the converse also held true. These findings, together with the apparent absence of synergism between the natural inducer and choline, led the authors to conclude not only that choline and the natural inducer act at different sites, but also that choline plays no part in the initiation of metamorphosis in the natural environment.

Three possible mechanisms concerning choline-mediated metamorphosis were proposed on the strength of the above study by Hirata & Hadfield (1986). First, that externally applied choline constitutes a precursor in the process of acetylcholine synthesis; second, that the choline acts on normally acetylcholine specific receptor sites; and third, that choline may perform a role in the synthesis of catecholamine neurotransmitters. Choline has previously been implicated in catecholamine synthesis in vertebrates (see Blusztajn & Wurtmann, 1983). This process involves increased acetylcholine secretion and the time period required for completion could be taken as a reasonable explanation for the lag period of 2-3 days observed between artificially and naturally induced metamorphosis in *P. sibogae* (Hirata & Hadfield, 1986).

#### Morphogenic Properties of Potassium

Table 3.2 illustrates that veliger metamorphosis (mean 42.9%) was successfully triggered in 5 of the 6 experiments in which, 19mM elevated potassium was administered. Experiment 5 showed 29mM potassium to be supra-optimal, inducing a non-significant 14.3% of veligers to metamorphose, compared to a significant 31.0% of veligers subjected to, 19mM potassium. This concurs with the findings of Todd *et al.* (1991) which established *A. proxima* larvae to metamorphose in response to an optimal dose of, 19mM potassium, with 29mM proving supra-optimal or inhibitory. The contrast between this and the absence of metamorphic response displayed by embryos is well illustrated by Fig.3.2. It is, however, apparent that Experiment 7 represents a notable exception to this trend, with embryos induced to metamorphose in similar proportions to veligers (means 64.5 and 58.2% respectively). This atypical result did not prove repeatable in subsequent bioassays, and possibly was attributable to inadvertent contamination of the potassium treatment by choline.

The failure both of, 19mM and 29mM potassium to induce intracapsular metamorphosis relative to the success of choline chloride was unexpected and the cause unknown. It is however possible that the lack of response evident in *A. proxima* on subjection to potassium may not be solely attributable to the insufficient development of appropriate metamorphic pathways or receptors. It may be that the potassium ions do not penetrate the primary egg capsule membrane in sufficient

quantities to elicit a metamorphic response, even in larvae competent to metamorphose in response to potassium.

Of particular interest in relation to this point are the results of Experiments 6 and 13 (see Table 3.1 and 3.2). The unhatched status of the spawn masses at the initiation of these experiments necessitated the use of artificially hatched veligers sibling to the embryos. Elevated potassium elicited significant, albeit low, levels of metamorphosis in these veligers (44.1 and 28.7% respectively) whilst having no apparent morphogenic effect on embryos. Veligers were of identical provenance (and therefore age) to the embryos, the only difference being that veligers had been physically freed from their egg capsules prior to natural eclosion. These results may be interpreted to imply support for the impermeability of the *A. proxima* egg capsule with respect to potassium, or, alternatively, to imply that whilst embryos remain physiologically incompetent to potassium, upon eclosion (whether artificial or natural) specific developmental events are triggered rendering the veligers sensitive to elevated potassium levels.

Whilst studies of the osmotic regulatory and physiochemical properties of gastropod egg capsule membranes have revealed considerable interspecific differences in permeability (Pechenik, 1982), there is much evidence that they are, to differing extents, freely permeable to inorganic ions (Pechenik, 1982; 1983; Losse & Greven, 1993a and b; but see Eyster, 1986). In a study of the lecithotrophic brooding species *Littorina saxatilis* Losse & Greven (1993a) established the egg membrane to be highly permeable to a range of ionic species and compounds including water, lead and phosphate ions, amino-acids and proteins of up to 40,000 Daltons. Moreover, *L. saxatilis* (like *A. proxima*) develops an embryonic shell, which led Losse & Greven (1993b) to reason that  $\text{Ca}^{2+}$  ions must pass through the egg membrane to facilitate shell formation.

A comparison of the induction agents used within this study reveals the potassium ( $\text{M}_w 39$ ) cation to be considerably smaller than the choline molecule ( $\text{M}_w 104$ ). The successful induction of intracapsular metamorphosis by choline is evidence of the membrane permeability toward this relatively larger molecule in *Adalaria proxima*. That the egg capsules of the gastropod *Nucella lapillus* display a greater permeability to the ions  $\text{Na}^+$  and  $\text{Cl}^-$  than to the larger organic molecule threonine ( $\text{M}_w 119$ ) (Pechenik, 1983) would perhaps imply that the capsule membrane of *A. proxima* be permeable to potassium.

Elevated levels of inorganic ions such as potassium are proposed to initiate metamorphosis by a process distinct from that of neuroactive agents such as choline. Natural external environmental stimuli are proposed to translocate to internal nervous stimuli and thereby initiate metamorphosis by depolarizing larval sensory cell membranes (Morse, 1985; Baxter & Morse, 1987). Elevated external concentrations of inorganic cations may act on invertebrate larvae by directly causing a depolarizing shift in the membrane potential of external receptor cells in the absence of the natural inducer, resulting in the initiation of metamorphosis (Baloun & Morse, 1984; Morse, 1985; Yool *et al.*, 1986; Pawlik, 1990; Rodriguez *et al.*, 1993). Todd *et al.* (1991) advanced the hypothesis that rather than initiating the process of metamorphosis by depolarizing external chemosensory cells potassium may instead act by directly affecting target tissues. The alternative proposal of membrane depolarization has, however, received extensive support in the literature. In their study of the response of the larval nervous system of *H. rufescens* to metamorphic stimuli Baloun & Morse (1984) proposed that metamorphosis was initiated by the depolarization of the membrane potential of external 'excitable' cells. Whilst metamorphic induction by excess potassium was unaffected by the ion-exchange blocker sulfonyl isothiocyanostilbene, induction was prevented by the potassium channel blocker tetraethylammonium, providing evidence for the role of potassium in depolarizing external excitable cells. Subsequent tests established that a reduction in external  $\text{Ca}^{2+}$  concentration resulted in inhibition of the metamorphic response and thus led Baloun & Morse to propose the existence of  $\text{Ca}^{2+}$  regulated potassium channels in larval receptor cells. The decrease in external  $\text{Ca}^{2+}$  may have resulted in decreased intracellular  $\text{Ca}^{2+}$ ; thus the passage of  $\text{K}^{+}$  necessary for initiation of metamorphic induction may have been prevented by the  $\text{Ca}^{2+}$  regulated membrane channels. However, no such blocking of potassium channels was displayed by larvae of both *P. sibogae* and *P. californica* when subjected to TEA, implying that in these species potassium may act through channels distinct from those mediating initiation of metamorphosis in *H. rufescens* (Yool *et al.*, 1986). That potassium channels are still responsible for signal transduction in this species can be explained by the presence of distinct potassium channels which are insensitive to external TEA (Yool *et al.*, 1986).

### Morphogenic Properties of the Natural Cue

Whilst the inductive capacity of many metamorphic cues may be dependent on larval contact with the substratum, others may be effective within the water column (Crisp, 1984; Hadfield, 1984). The natural metamorphic cue of *A. proxima* is one such non-contact cue, successful metamorphosis requiring merely the presence of the adult prey *Electra pilosa* (Thompson, 1958a) or water previously 'conditioned' by it (Lambert & Todd, 1994). The water-borne nature of the cue therefore facilitated the investigation of competence in embryos still enclosed within the primary egg capsule.

*Electra pilosa* conditioned sea water (CSW) was effective in triggering significant levels of extra-capsular (veliger) metamorphosis in five of the eight experiments assayed (see Table 3.2). Considerable between-experiment variation in response was apparent (Fig.3.3). Such variability of *Adalaria proxima* larvae in response to the natural cue is not atypical, and indeed has been previously reported (Todd *et al.*, 1991; Lambert & Todd, 1994). The corresponding embryonic responses were entirely negative, the maximum of 6.1% of intracapsular metamorphosis apparent in Experiment 8 proved to be non-significant (see Table 3.1). The lack of response to the natural cue by encapsulated embryos illustrates that the phenomenon of intracapsular metamorphosis is not an indication of incipient poecilogony in this species and, whilst supplying pertinent physiological information, is of little ecological relevance.

### Morphogenic Properties of Other Neuroactive Compounds

In addition to those compounds previously established to be effective metamorphic cues in *A. proxima*, the neuroactive agents dopamine, L-DOPA and GABA were screened for morphogenic activity with respect to both embryo and veliger *A. proxima*.

Neither L-DOPA or the catecholamine dopamine were effective in inducing metamorphosis in embryos or hatched veliger larvae competent with respect to choline (see Table 3.5). Dopamine was assayed over the concentrations  $10^{-2}$ - $10^{-5}$ M, a range established to trigger metamorphosis in other marine invertebrates. Velar regression (reduction or loss of velar lobes), indicative of partial



metamorphosis, has been observed in response to dopamine in other opisthobranch species (Hadfield, 1984), but was not evident in *A. proxima*

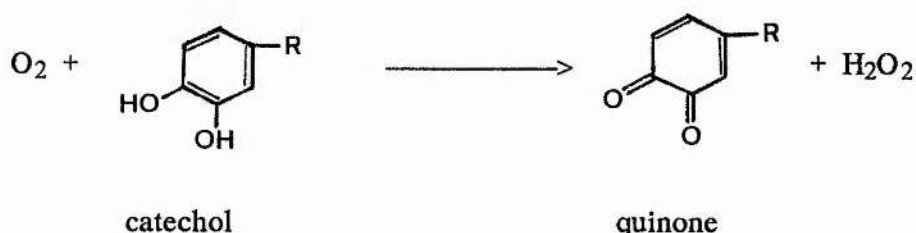
Molluscan larvae have been the focus of many studies of the putative mechanism(s) by which catecholamines may mediate metamorphosis. In a study of L-DOPA and catecholamine-induced settlement and metamorphosis of *Crassostrea gigas* and *Crassostrea virginica*, Coon *et al.* (1985) successfully induce pediveligers by administration of L-DOPA, and metamorphosis (with the absence of settlement behaviour) in response to noradrenalin and adrenalin. Whilst both species proved sensitive to catecholamines, the responses differed in that *C.gigas* displayed a higher, and more consistent, rate of metamorphosis than did *C.virginica*, and epinephrine was found to be more potent, and elicit a faster response than norepinephrine. The fact that epinephrine proved incapable of inducing pre-competent larvae, and that metamorphosis proceeded without the usual associated settlement response, led the authors to conclude that adrenalin acts at a later stage of, or on discrete pathway to, that involved in settlement.

Further support of the hypothesis of catecholamines mediating metamorphosis by providing the requisite endogenous signals for morphogenesis to proceed derives from Coon & Bonar (1986), Coon *et al.* (1986), Coon & Bonar (1987a & b) and Bonar *et al.* (1990). Coon & Bonar (1986) quantified the temporal profile of endogenous catecholamines present in larvae of *C.gigas*. Whilst dopamine was present in larvae of all ages, and epinephrine could not be determined in significant amounts at any time, perhaps the most noteworthy finding was that norepinephrine levels rose significantly from hatching to competence (0.062pg/ $\mu$ g up to 1.08 pg/ $\mu$ g protein). The authors proposed that norepinephrine may function by acting directly on target tissue (the Peripheral Receptor Theory) or by causing the release of a hormone which in turn would act on target tissue (the Centralized Receptor Theory). Further support for the importance of catecholamines in this process was supplied by Coon & Bonar (1987) who were able to pharmacologically identify specific epinephrine and norepinephrine receptors in *C.gigas* larvae as vertebrate-type  $\alpha_1$ -adrenoreceptors. Information provided in previous studies was integrated by Bonar *et al.* (1990) who proposed a more detailed model of the behavioural and morphogenetic processes of *Crassostrea*. Settlement behaviour, initiated by dopamine derived from exogenously applied L-DOPA, was proposed to constitute a separate pathway to morphogenesis and to act upon a dopaminergic receptor mediated path. Conversely, metamorphosis, was

postulated to be triggered by both noradrenalin and adrenalin and involve an andrenergic receptor mediated pathway .

Whereas dopamine has been established to exhibit morphogenic properties in other marine invertebrate larvae (Burke, 1983), the lack of response exhibited by *A. proxima* cannot be regarded as atypical. Limited and sporadic responses have been reported for several species in response to dopamine. The nudibranch *P. sibogae*, for example, when subjected to  $10^{-5}$ - $10^{-3}$ M dopamine over 4 days, showed a maximum of just 31% metamorphosis on treatment with the optimum dose ( $10^{-4}$ M). Similarly, the activity of dopamine on the gastropod *Ilyanassa obsoleta* has been described as 'minor' (Levantine & Bonar, 1986).

Catecholamines are unstable and rapidly oxidize in seawater (Jensen & Morse, 1984; Pawlik, 1990; Pires & Hadfield, 1991; Waite, 1992; Haws & DiMichele, 1993), following the general reaction:



(From Pires & Hadfield, 1991)

The relevance of this oxidation to metamorphic inducing activity has been addressed in several studies (Hadfield, 1984; Chevolot *et al.*, 1991; Pires & Hadfield, 1991; Haws & DiMichele, 1993). It has been proposed that it is not the catecholamines that produce a settlement or metamorphic response, but rather it is their oxidative breakdown products (Chevolot *et al.*, 1991; Pires & Hadfield, 1991 but see Pawlik, 1990 and Haws & DiMichele, 1993). Pires & Hadfield (1991) failed to induce any metamorphic response on administration of a range of fresh, unoxidized catecholamines (epinephrine, norepinephrine, isoproterenol, dopamine, dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid and catechol), yet successfully induced partial metamorphosis using these same aged (and therefore



oxidized) catecholamines. Whilst not negating the possible activity of catecholamines, these results do support the hypothesis that the reactive species (OH<sup>-</sup>) derived from hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), an oxidative breakdown product of catecholamines, may have a function.

Chevolot *et al.* (1991) established that both L-DOPA and epinephrine produced significant amounts of metamorphosis in competent larvae of the bivalve *Pecten maximus*, and, like Pires & Hadfield (1991) deduced the oxidation products to constitute the active component. Unlike Pires & Hadfield (1991) who proposed hydrogen peroxide to be the active fraction, however, Chevolot *et al.* (1991) suggested the active oxidative product to be a quinone, or to possess a quinone-like structure (see generalized reaction above).

In an attempt to minimize oxidation of adrenalin in metamorphic trials Haws & DiMichele (1993) supplemented 10<sup>-4</sup>M test solutions with ascorbic acid and used only short exposure periods (4 hours) in the dark. The metamorphic responses of both *Crassostrea gigas* and *C. virginica* pediveligers were similar (93-96%) and, indeed, improved over the variable response of 10<sup>-4</sup>M adrenalin previously reported by Coon *et al.* (1986) for both *C. gigas* and *C. virginica*. These results, albeit on different species, clearly do not concur with the findings of Chevolot *et al.* (1991) and Pires & Hadfield (1991). The hypotheses that metamorphosis is mediated by either catecholamines or their oxidised products may not therefore be mutually exclusive, and the response may exhibit some degree of species specificity.

Rapid discolouration of the dopamine treatments used within this study was observed and indicated the presence of oxidative breakdown products. Oxidation of the strongest (10<sup>-3</sup>M) treatment became apparent within 1 hr at 10°C, whilst at the termination of exposure time (7 hrs) the 10<sup>-4</sup>M treatment had also become discoloured. This was interpreted to indicate that larvae had been exposed to both the catecholamine initially, and oxidative breakdown products latterly.

The neurotransmitter GABA, assayed over the range 10<sup>-2</sup>-10<sup>-7</sup>M, proved effective in inducing metamorphosis in hatched *Adalaria proxima* veligers. The optimal, and indeed only effective, dose was established to be 10<sup>-3</sup>M, which was capable of inducing 23% metamorphosis after 4 days. Todd *et al.* (1991), demonstrated choline to exhibit a latent period of 24 hrs, resulting in metamorphosis within 2 days, although this may vary slightly, due possibly to larval age related

effects (Lambert & Todd, 1994). The rate of GABA-mediated metamorphosis displayed in Fig. 3a implies that this may exhibit a greater latency period relative to choline, the greatest response occurring as it does after 3-4 days. The maximum response of 23%, although significant, was significantly lower than that triggered by choline.

Larvae of another nudibranch species, *Phestilla sibogae*, have been reported to exhibit a similar response to GABA. For assays over the concentration range  $10^{-3}$ - $10^{-4}$ M, Hadfield (1984) found  $10^{-4}$ M to elicit an optimal response of just 30% metamorphosis. The frequently unsuccessful and consistently highly variable nature of this response led him to describe GABA as essentially inert to *P. sibogae* larvae. As was the case for *A. proxima*, Hadfield (1984) found that whilst *P. sibogae* responded to other artificial inducers, including GABA, choline appeared to be by far the most effective.

In contrast to the veliger response, GABA did not induce metamorphosis among *Adalaria proxima* embryos. Using the same rationale applied to other morphogenic cues successful in inducing only veliger metamorphosis in this study, the failure of GABA may be attributable to either the physiological inability of embryos to detect or respond to GABA, or to the impermeability of the egg capsule wall. It is interesting to note, however, that the size of the GABA molecule, at  $M_w$  103, is very similar to that of choline ( $M_w$  104) and would therefore not necessarily be expected to be precluded from penetration of the egg capsule membrane on the basis of its molecular size. Were the free passage of GABA through the egg capsule membrane to be the case, these results would imply choline to be effective at a later stage, or on a discrete pathway, than to GABA.

The mechanism of GABA-mediated induction of metamorphosis has been most extensively investigated in the red abalone *Haliotis rufescens* (Baloun & Morse, 1984; Morse, 1985; Trapido-Rosenthal & Morse, 1986b, Fenteany & Morse, 1993). In a study of excitable cells in the larval nervous system of *H. rufescens* Baloun & Morse (1984) found that induction of metamorphosis by GABA was dose dependent and inhibited by both decreased external potassium ion levels and by increased external calcium ion levels. In addition, whilst GABA induced metamorphosis was inhibited by the neuropharmacological anion exchange blocker sulfonyl isothiocyanostilbene, and unaffected by tetraethylammonium, the converse was true regarding induction by elevated potassium concentration. This

experimental evidence was interpreted by Baloun & Morse (1984) to indicate that both GABA and elevated potassium mediate metamorphosis by depolarization; this would involve cross membrane transport of specific ions, but they apparently act at different, mutually exclusive, sites. The definite site at which GABA acts is unknown (Baloun & Morse, 1986b). The hypothesis that GABA effects metamorphosis by depolarization of external chemoreceptor cells (Baloun & Morse, 1984; Morse, 1985; Trapido-Rosenthal & Morse, 1986b) has received less support in the literature than the alternative that GABA-induced metamorphosis is mediated by internal nervous stimulation of relevant neurones (Hirata & Hadfield, 1986; Pawlik, 1990; 1992). GABA has been documented to be present in the crustose coralline algal species demonstrated to induce settlement and metamorphosis in *H. rufescens* (Morse *et al.*, 1979). A study by Johnson *et al.* (1991) could, however, detect no free GABA in cell-free extracts of coralline algae (or associated bacteria), and concluded on this basis that GABA had no ecological significance as a metamorphic inducer.

#### Cue specific competency

The results of the present study suggest that *Adalaria proxima* progeny display cue-specific competency. Embryos attain the ability to fully metamorphose in response to choline whilst remaining refractive to treatment both with the natural cue and elevated potassium levels sufficient to successfully induce metamorphosis in veliger larvae. Table 3.4 shows that whilst a significant 42.9 % (n=5) and 28.9% (n=5) of veligers were triggered to metamorphose in response to, 19mM potassium and the natural cue respectively, the corresponding embryo responses were non-significant at just 3.9% and 0.4%. Choline was, therefore, the only cue established to be capable of eliciting metamorphosis in unhatched, eclosed embryos.

The phenomenon of cue-specific competence was first documented by Pechenik & Gee (1993) in hatched larvae of the prosobranch gastropod *Crepidula fornicata*. Using both the natural cue (adult conditioned sea water) and 20mM potassium excess, the authors found larvae attained competence to respond to elevated potassium 12-24 hours prior to the attainment of competence with respect to the natural cue. Whilst this developmentally earlier response to potassium was interpreted to indicate a different site of action for potassium than that of the natural cue, it is not clear whether potassium and natural cue-mediated metamorphosis

occur through the same or different pathways. It may be that potassium acts at a later stage in the same pathway as the natural cue, although potassium and natural cue-mediated metamorphosis in *C.fornicata* have been previously reported to be morphologically distinct (Pechenik & Heyman, 1987), which would infer the presence of different pathways.

The present study did not follow competence and latency periods on the fine time scale (twice daily) of Pechenik & Gee (1993), and employed instead the discrete states of unhatched embryo or veliger. Consequently, beyond establishing that neither potassium or the natural cue trigger intracapsular metamorphosis, little more can be implied concerning the relative attainment of competence with respect to either cue.

In contrast, the effectiveness of choline in triggering intracapsular metamorphosis compared to the inactivity of both elevated potassium levels and the natural cue provides information on the ontogeny of metamorphic pathways in *A. proxima*. For example, if two cues act upon the same site in the metamorphic pathway, it would be logical that competence toward these cues be attained simultaneously. Any differential attainment of competence would therefore imply different sites of action, although not necessarily different metamorphic pathways.

#### The Differential Morphogenic Natures of Choline and Potassium

Todd *et al.* (1991) investigated possible synergistic or antagonistic effects between the two artificial inducers potassium and choline in *Adalaria proxima*, providing information concerning the nature of the inductive processes triggered by choline and potassium respectively. This was achieved by interaction experiments in which larvae were subjected to combinations of these artificial inducers. Supra-optimal concentrations (29mM) of potassium were found to inhibit choline-induced metamorphosis. This led the authors to postulate that potassium and choline act either upon separate pathways, or differing points in the same pathway. Further support for the former hypothesis was inferred by the outcome of timed exposure trials. Competent veliger larvae were immersed in optimal concentrations of either choline (10mM) or potassium (19mM) test solutions for discrete periods between 1 hr and 5 days. Whilst continuous immersion in potassium was required to elicit a response, a mere 1-2 hrs exposure to choline was sufficient to initiate

metamorphosis. If the successful passage of potassium cations through the primary egg capsule membrane is assumed then the differential responses of embryos displayed toward potassium and choline in this study would lend further support to the hypothesis that potassium and choline mediated metamorphosis are exclusive processes.

#### Differential Morphogenic Natures of Choline and Natural Cue

The complete lack of structural similarity between effective neuroactive inducers and those few natural metamorphic inducers which, to date, have been identified has been used to support the view that natural and artificial cues mediate metamorphosis in dissimilar fashions (see review by Pawlik, 1992). Support for the differential nature of action between choline and natural inducer is supplied by the findings of Pawlik (1990) in his investigation of metamorphic induction in larvae of the polychaete *Phragmatopoma lapidosa californica*. Metamorphosis displayed a lag phase of 12-24 hrs in response to choline, relative to that initiated by the natural inducer. Further, metamorphosis in response to choline appeared abnormal, (manifested by the absence of larval settlement and tube building) implying the bypassing of a step in the normal morphogenetic pathway. In comparing the processes involved in artificially and naturally triggered metamorphosis, therefore, the experimental evidence of Pawlik concurred with that of Hirata & Hadfield (1986) in indicating that choline acted directly upon the nervous system and not on external receptors associated with the natural cue. Whilst the site of choline-mediated metamorphosis within larvae of *A. proxima* is unknown, Todd *et al.* (1991) considered the alternative hypotheses that choline may bind to external receptors or be actively taken up through the epithelial (or endothelial) membranes.

Assuming the successful passage of the natural cue across the capsule membrane, the differential nature of the embryo responses to choline and conditioned sea water indicate that these cues act at different sites and/or by distinct pathways. Moreover, the prior attainment of competence with respect to choline relative to the natural cue would tend to infer that choline acts before the functional completion of external chemoreceptors. Support is thereby supplied to the later hypothesis of Todd *et al.* (1991) that choline-mediated metamorphosis may bypass



external chemoreceptors and instead proceed by active uptake across the epithelial or endothelial membrane.

The processes of artificially and naturally induced metamorphosis in *Adalaria proxima* were examined in a comprehensive study by Todd *et al.* (1991). Whilst interaction experiments of the type reported in Hirata & Hadfield (1986) between choline and the natural inducer were not performed for *A. proxima*, inference may be drawn from the stated latency periods. The inducers (natural cue, 19mM potassium and 10mM choline) did not differ in the rate at which metamorphosis was induced. Metamorphic induction displayed a minimal period of 24 hours after initial exposure to the cue and thereafter metamorphosis proceeded to completion within a further 24 hours, whether the agent was the natural inducer, potassium, or choline. This is perhaps noteworthy in the light of the hypotheses proposed by Hirata & Hadfield (1986) concerning the mechanisms of action in natural cue and choline induced metamorphosis. Both *Phestilla sibogae* and *Phragmatopoma lapidosa californica* display a lag period in response to choline relative to their respective natural inducers, this time delay being consistent with, although not establishing, the postulated role of choline in the synthesis of catecholamines neurotransmitters. The absence of any such difference in latency period between larvae of *A. proxima* successfully induced to metamorphose on subjection to either the natural cue or choline chloride contrasts markedly with the disparate latency periods of inducers documented by both Hirata & Hadfield (1986) and Pawlik (1990). Todd *et al.* (1991) make the point that absolute rate comparisons are invalid when considering the tropical nudibranch *P. sibogae* and the temperate nudibranch *A. proxima*. Whilst this undoubtedly is the case, valid comparisons can be drawn from the relative differences in metamorphic induction rates of inducers within each species. The absence of a lag period between naturally induced and choline induced metamorphosis apparent in *A. proxima* does not preclude the validity of any of the three hypotheses on the mechanism of choline-mediated metamorphosis put forward by Hirata and Hadfield (1986). That the inductive period for both natural and choline induced metamorphosis are similar, however, does perhaps question the hypothesis that choline performs a role in the synthesis of catecholamine neurotransmitters, at least in *A. proxima*.

The disparate interspecific responses of invertebrate larvae toward artificial inducers may be indicative of the evolution of different metamorphic mechanisms (Hadfield, 1984). For example, Hadfield (1984) has remarked on the contrary

nature of artificially induced metamorphosis in the gastropods molluscs *Phestilla sibogae* and *Haliotis rufescens*. Whilst *P. sibogae* may be successfully induced to metamorphose in response to choline (Hadfield, 1978; Hirata & Hadfield, 1986), *H. rufescens* cannot (Morse *et al.*, 1979), and, conversely, whilst *H. rufescens* is reliably induced to metamorphose in response to GABA (Morse *et al.*, 1979; Morse & Morse, 1984; *P. sibogae* is not (Hadfield, 1984). This study shows that *A. proxima*, like *P. sibogae*, displays minimal sensitivity towards GABA relative to the high degree of successful metamorphosis obtainable by the administration of choline or potassium (Todd *et al.*, 1991). For these reasons, the metamorphic mechanisms of *A. proxima* may more closely be linked to the nudibranch *P. sibogae* than the prosobranch *H. rufescens*.

### Conclusion

In summary, the results of this study establish that *A. proxima* does indeed exhibit cue-specific competency. Artificial induction agents have previously been regarded as reliable practical indicators of competence in this and other marine invertebrate species (Pechenik & Heyman, 1987; Bonar *et al.*, 1990; Coon *et al.*, 1990; Todd *et al.*, 1991). The findings of this study therefore echo the conclusion of Pechenik & Gee (1993) that metamorphic competence in the laboratory must be defined in relation to the relevant cue.

Comparison of the resultant cue-specific responses provides information concerning choline-mediated metamorphosis and the ontogeny of metamorphic pathway(s) in *Adalaria proxima*. Assuming egg capsule membrane permeability towards all cues, the attainment of competence towards choline prior to elevated potassium and the natural cue would supply additional support that the site of choline-mediated metamorphosis be distinct from that of both elevated potassium and the natural cue, although it does not qualify whether choline acts at a later point in the same pathway to, or on an exclusive pathway to, excess potassium and the natural cue. Natural cues are proposed to initiate metamorphosis by the depolarization of external larval receptor cell membranes (Morse, 1985; Baxter & Morse, 1987). In a similar fashion excess potassium is proposed to act by depolarizing the membrane potential of sensory cells (Baloun & Morse, 1984; Morse, 1985; Yool *et al.*, 1986; Pawlik, 1990; Rodriguez *et al.*, 1993). The embryonic insensitivity towards excess potassium and the natural cue relative to the



efficacious nature of choline may then imply the choline-mediated metamorphic pathway to be functionally complete at an ontogenetically earlier stage than the formation of external larval chemoreceptors.

## CHAPTER 4

### THE NATURAL METAMORPHIC CUE

#### 4.1 INTRODUCTION

The demonstration that invertebrate larvae respond to environmental stimuli led to the realization that larval settlement was not, as previously regarded, simply a random process (Colman, 1933; Young, 1937) but rather, was influenced by active substratum selection (Crisp & Meadows, 1963; Crisp, 1967; Meadows & Campbell, 1972 a & b; Crisp, 1974; Chia, 1978; Strathmann & Branscomb, 1979; Le Tourneux & Bourget, 1988). Non-random settlement and metamorphosis are mediated by ecologically relevant cues associated with preferred settling substrata (Hadfield, 1978 a; Coon *et al.*, 1985) such that metamorphosis will occur in favourable juvenile sites with relatively high survival probability to adulthood (Burke, 1983; Hadfield & Scheuer, 1985; Pennington & Hadfield, 1989).

Whilst in some species both settlement and metamorphosis may be mediated by the same cues (Hadfield, 1978a; Inestrosa *et al.*, 1988), these processes may be considered physiologically distinct (Chia & Koss, 1988). Settlement is regarded as a reversible and repeatable behavioural response to exogenous environmental cues (Burke, 1983; Coon *et al.*, 1985; Chia & Koss, 1988; Bonar *et al.*, 1990; Tamburri *et al.*, 1992). Like metamorphosis, settlement may be triggered both by physical and biological exogenous stimuli such as surface contour and texture (Crisp & Barnes, 1954), light intensity (Sebens, 1983; Morse, 1991) and current velocity (Sebens, 1983; Pawlik *et al.*, 1991). This subject of settlement-inducing stimuli has been addressed in several comprehensive review articles (Thorson, 1957, Meadows & Campbell, 1972 a, b; Crisp, 1974; 1976; Chia & Rice, 1978; Pawlik, 1992; Rodriguez *et al.*, 1993) and is not the focus of the present study.

In contrast, metamorphosis is regarded as constituting an irreversible (but see Richmond, 1985) developmental step involving fundamental morphological change (Hadfield & Scheuer, 1985; Chia & Koss, 1988; Bonar *et al.*, 1990; Tamburri *et al.*, 1992) and resulting in an ontogenic niche shift from larval to benthic juvenile form (Burke, 1983; Werner, 1986; Rowe & Ludwig, 1991). The terms settlement and metamorphosis are not however rigidly defined in the literature and within several contexts the term settlement may be used to encompass the entire shift from planktonic larval to benthic juvenile phase (Hadfield, 1978; Pawlik, 1992). Within the confines of the present study, larval settlement will be considered distinct from metamorphosis, and it is upon the later that the present emphasis is placed.

The absence of an intrinsic morphogenic trigger in marine invertebrate larvae necessitates the presence of exogenous environmental stimuli to mediate initiation of metamorphosis (Hofmann & Brand, 1987). This study is concerned with those exogenous biological stimuli which have been demonstrated to mediate larval metamorphosis. Morphogenic cues emanate from a range of sources within the marine environment. Cues may be associated with the presence of conspecifics, algae, microbial films or adult prey species, each of which will be addressed below;

## Origins Of Morphogenic Cues

### Morphogenic Cues From Conspecifics

Gregarious settlement -that is, settlement and metamorphosis in response to conspecifics -is regarded as a relatively common phenomenon among marine invertebrates, and especially for sessile hard-bottom community species (Pawlik, 1992). The literature has been reviewed by several authors (Crisp, 1974; Burke, 1986; Pawlik, 1986; Coon *et al.*, 1986) and morphogenic stimuli may originate from the presence of other adults (Knight-Jones, 1953, Burke, 1984) or juveniles (Cole & Knight-Jones, 1949, Leitz & Lange, 1991).

Gregarious settlement has been established to occur in many classes higher taxa, including; barnacles (Knight-Jones, 1953, Crisp, 1979; Chia, 1989; Raimondi, 1991), polychaetes (Knight-Jones, 1953, Jensen & Morse, 1984; Pawlik, 1986, 1988; Raimondi, 1991), echinoids (Chia, 1989; Highsmith, 1982; Burke, 1984; Pearce & Scheibling, 1990), bivalves (Veitch & Hidu, 1971; Eyster

& Pechenik, 1987), and gastropods (Levantine & Bonar, 1986; McGee & Targett, 1989).

Morphogenic cues are proposed to act in many, although not all, species exhibiting gregarious distribution. For example, of the 35 species reviewed by Burke (1986) and reported to exhibit gregarious settlement in over 50% of cases chemical cues were implicated. The metamorphosis of larvae in response to cues emanating from conspecifics results in aggregations of adults, and the adaptive advantages conferred by such groupings are several; 1. The probability of survival to adulthood is increased by the colonization of established favourable habitats (Crisp, 1974; Jensen, 1989, 1991; Highsmith, 1982; Tamburri *et al.*, 1992). 2. Both free-spawning species and those which undergo internal fertilization benefit from proximity to potential mates (Crisp, 1979; Pennington, 1985; Pawlik, 1986; Tamburri *et al.*, 1992). The negative aspects of such aggregated settlement include increased mortality due to intense competition for food (Crisp, 1979) and available space as well as ingestion or removal of juveniles by adult conspecifics (Pawlik, 1992).

There are no known examples within the literature of a nudibranch mollusc exhibiting metamorphosis in response to conspecifics or the products thereof. The sole example within the Opisthobranchia is provided by the cephalaspidean *Haminoea callidegenita*. Both embryos and veliger larvae have been demonstrated to metamorphose in response to a water soluble substance emanating from the egg mass jelly (Gibson & Chia, 1989a, 1994). Whilst this does not result in gregarious settlement as such, Gibson & Chia (1994) postulate that the advantages of employing such a morphogenic cue are similar by ensuring both the colonization of suitable juvenile habitats and recruitment to the parent population.

#### Morphogenic Cues From Algae

Many herbivorous invertebrate species are induced to settle and metamorphose by the algal species upon which the adults or juveniles graze (Morse, 1992; Pawlik, 1992; Rodriguez *et al.*, 1993). Members of the Rhodophyta, Chrysophyta and Phaeophyta have been demonstrated to produce morphogenic substances. The subject has been extensively reviewed by Scheltema (1974) and, more recently, by Morse (1992).

Many epiphytic species have been shown to preferentially settle and metamorphose on specific macroalgal species (Ryland, 1974; Scheltema, 1974). Such is the case for the bryozoans *Alcyonidium hirsutum*, *Alcyonidium polyomm* and *Flustrellidra hispida* which settle preferentially upon the fucoid *Fucus serratus* (Ryland, 1958, 1974). Larvae of the epiphytic hydrozoan *Coryne uchidai* preferentially settle and metamorphose on *Sargassum tortile* (Kato *et al.*, 1974).

Settlement association with macroalgae is not however confined to epiphytes and is displayed by many genera. For example, Davies & Stoner (1994) established larvae of the Queen Conch *Batophora oerstedii* to show a maximal metamorphic response relative to conspecifics, sediment and macroalgae to occur in response to the juvenile food source *Thalassia testudinum*. Likewise, larvae of the bivalve *Mytilus edulis* metamorphose in response to filamentous red algae (Eyster & Pechenik, 1987).

The non-genticulate crustose coralline algae grazed upon by a large range of invertebrate herbivores have been regarded as a particularly important source of settlement and morphogenic stimuli (see the reviews of Burke, 1983; Morse, 1992; Rodriguez *et al.*, 1993). Table 4.1 shows the considerable range of invertebrate species induced to metamorphose in the presence of crustose coralline algae.

The identity of morphogenic substances produced by crustose coralline algae has been the focus of much attention. In their study of the echinoderms *Pseudocentrotus depressus* and *Anthocidaris crassispina*, Kitamura *et al.* (1993) isolated the active fraction of the coralline alga *Corallina pilulifera* by silica gel chromatography. The metamorphosis evident in response to the alga was found to be attributable to the fatty acids eicosapentaenoic acid (20:5) and arachidonic acid (20:4).

Perhaps the most extensively researched example of investigation into a crustose coralline alga produced morphogenic cue is provided by Morse and co-workers for the gastropod *Haliotis rufescens* (Red Abalone). Since the preferential settlement and metamorphosis of *H. rufescens* larvae in response to *Lithothamnium* sp. was first documented (Morse *et al.*, 1979), much information concerning the specificity and identity of the cue has been sought (Morse *et al.*, 1980; Morse & Morse, 1984a, b; Morse *et al.*, 1984; Morse, 1985). *H. rufescens* larvae will settle and metamorphose on contact with several ecologically relevant crustose coralline algal species (e.g. *Lithophyllum* and *Hildenbrandia* sp.) in addition to

	Invertebrate Species	Crustose Coralline Algal Species Providing Metamorphic Cue	Authoritory
Echinodermata	<i>Pseudocentrotus depressus</i>	<i>Corallina pilulifera</i>	Kitamura <i>et al.</i> (1993)
	<i>Anthocidaris crassispina</i>	<i>Corallina pilulifera</i>	Kitamura <i>et al.</i> (1993)
	<i>Strongylocentrotus droebachiensis</i>	<i>Lithothamnion glaciale</i> <i>Phymatolithon laevigatum</i> <i>Phymatolithon rugulosum</i> <i>Corallina officinalis</i>	Pearce & Schiebling (1990)
	<i>Strongylocentrotus purpuratus</i>		Rowley (1989)
Cnidarians	<i>Alcyonium siderium</i>	<i>Lithothamnion glaciale</i> <i>P. rugulosum</i> <i>Waernia mirabilis</i>	Sebens (1983)
	<i>Agaricia humilis</i>	Unspecified	Morse <i>et al.</i> (1988)
	<i>A. tenuifolia</i>	Unspecified	Morse <i>et al.</i> (1988)
	<i>A. agaricites</i>	Unspecified	Morse <i>et al.</i> (1988)
Asteroidea	<i>Acanthaster planci</i>	<i>Lithothamnium pseudosorum</i>	*Henderson & Lucas (1971) Johnson <i>et al.</i> (1991a)
Gastropoda	<i>Haliotis rufescens</i>	<i>Lithothamnium sp.</i> <i>Lithophyllum sp.</i> <i>Hildenbranchia sp.</i>	Morse <i>et al.</i> (1979, 1980) Morse <i>et al.</i> (1984a & b)

\* but see Johnson & Sutton (1994).



*Lithothamnion* sp. (Morse *et al.*, 1979a, 1980; Morse & Morse, 1984) although not in response to the diatoms, phytoplankton, bacteria (Morse *et al.*, 1979; 1980c) and green or brown algae tested (Morse & Morse, 1984a). The active cue molecules were established to be present only at the algal surface and Morse & Morse (1984a) proposed fresh inducer molecules to be continually provided through the sloughing action of limpet grazing. The morphogenic properties of the artificial agent GABA ( $\gamma$ -amino butyric acid) have been previously reviewed (see Section 3.1 & 3.4), and it was initially postulated that the natural cue was protein-associated GABA on the algal surface. Subsequent investigation however, showed other Rhodophytes (*Porphyra* sp.) and the homogenized extracts of cyanobacteria to contain active morphogenic fractions and gel filtration and ion-exchange chromatography established the cue to be associated with algal biloproteins, with a size of MW 640-1,250 Da (Morse & Morse, 1984b, Morse *et al.*, 1984). Whilst it became apparent that the natural induction agent was not GABA, thereafter the identical effect of the natural cue and of GABA on *H. rufescens* larvae resulted in the natural cue being termed a 'GABA-mimetic molecule'. Radioactive labelling of known GABA-receptors has shown the natural morphogenic agent does indeed mimic GABA by binding to GABA specific receptors (Morse, 1990). There is to date, however, little evidence to support the presence of any further similarity between the natural cue molecule and GABA, save that they both induce metamorphosis in *H. rufescens* larvae. This terminology of a 'GABA-mimetic' natural inducer is therefore, not perhaps the most appropriate and may create the opportunity for misunderstanding and the inappropriate association of the two molecules.

#### Morphogenic Properties of Microbial Films

The settlement inducing and morphogenic properties of microbial films upon marine invertebrates are well established (see reviews by Meadows & Campbell, 1972a; Crisp, 1974; Scheltema, 1974; Bonar *et al.*, 1986; Pawlik, 1992; and Rodriguez *et al.*, 1993). The effective morphogenic properties have been documented in larvae across a range of taxa such as polychaetes (Kirchmann *et al.*, 1982; Maki & Mitchell, 1985), echinoderms (Johnson *et al.*, 1991; Johnson & Sutton, 1994), scyphozoa (Hofmann & Brand, 1987), echinoids (Cameron & Hinegardner, 1974; Pearce & Scheibling, 1991), bryozoans (Maki *et al.*, 1989), hydroids (Leitz *et al.*, 1994) and bivalves (Weiner *et al.*, 1985; Bonar *et al.*, 1990; Fitt *et al.*, 1990).

Bacteria may provide morphogenic stimuli by several means, none of which are necessarily mutually exclusive. It may be that larval contact with the characteristic surface texture of a microbial film is required (Cameron & Hinegardner, 1974; Kirchmann *et al.*, 1982). Alternatively bacteria may provide soluble cue substances by either direct secretion (Weiner *et al.*, 1985; Hofmann & Brand, 1987; Fitt *et al.*, 1990) or by releasing soluble compounds resulting from the enzymatic degradation of organic substances (Hofmann & Brand, 1987).

An example of contact with a microbial film eliciting metamorphosis is provided by larvae of the spirorbid polychaete *Janua brasiliensis*. Larval metamorphosis has been demonstrated on contact with both cultured films of bacteria isolated from the chrysophyte *Ulva lobata* (Kirchmann *et al.*, 1982) and the bacterium *Pseudomonas marina* (Maki & Mitchell, 1985). The active morphogenic agent was proposed to be bacterial exopolysaccharides (Kirchmann *et al.*, 1982). Maki & Mitchell (1985) later established that lectins on the larval surface bind to a D-glucose containing molecule in the exopolymer of the bacteria, providing further evidence of the contact nature of the cue.

A combination of settlement and metamorphic stimuli is utilized in the association between the bivalve *Crassostrea virginica* and the bacterium *Alteromonas colwelliana*. Weiner *et al.* (1985) first established *A. colwelliana* to be closely associated with oysters and their habitats, and identified the compound produced by this bacteria as melanin. The authors proposed that the bacteria adhere to the substratum by an acetic polysaccharide exopolymer and exude L-DOPA (a melanin precursor) and melanin. Whilst the exudates act as an attractant it is the microbial film itself that provides the morphogenic stimulus (see also Coon *et al.*, 1990b). Fitt *et al.* (1990) later established bacterial supernatants of both *A. colwelliana* and *Vibrio cholerae* to induce metamorphosis, the cue being a small (<300 Da) molecule.

Johnson *et al.* (1991a) have proposed that the morphogenic activity evident on coralline algal surfaces discussed above may not be algal in origin, as is widely assumed, but rather may be attributable to a characteristic surface microfauna. Few studies concerning the morphogenic properties of coralline algae have, to date, thoroughly addressed the possible influence of surface bacteria. Morse *et al.* (1984) found that whilst homogenized extracts of bacteria induced metamorphosis among *Haliotis rufescens* larvae, intact living bacteria did not. This led the authors to

dismiss the rôle of bacteria in the metamorphic induction of *H. rufescens* in the field.

More recent investigations into the rôle of coralline algae in invertebrate metamorphic induction (Johnson *et al.*, 1991a, b, Johnson & Sutton, 1994) have however proved elucidatory. Marine invertebrate larvae demonstrate specificity, albeit to varying degrees, in their metamorphic requirements in response to coralline algae. The rationale proposed by Johnson *et al.* (1991a) -that surface bacteria, and not the coralline alga itself act as morphogenic agents -therefore requires the presence of specific and characteristic microfaunal assemblages on coralline algal surfaces. This was tested experimentally by comparison of the bacterial assemblages of two South African coralline algal species, *Sporolithon* sp. and *Clathromorphum* sp., sea water and glass slides (Johnson *et al.*, 1991b). Not only was a difference established between the coralline algal species, but also a difference in bacterial populations was apparent between the coralline algae and the other treatments (sea water and glass slides). These findings confirmed the existence of characteristic and species specific bacterial assemblages on coralline algae.

Further investigation concerned the Crown-of-Thorns starfish *Acanthaster planci* which may be induced to settle and metamorphose in response to the coralline alga *Lithothamnium pseudosorum* (Johnson *et al.*, 1991a). Utilizing shards of coralline algae and associated bacteria, Johnson & Sutton (1994) presented evidence for the first time that metamorphosis was not exclusively attributable to coralline algae, but rather to the bacterial assemblage associated with it. Further information concerning the relationship between coralline algae, associated bacteria, and *A. planci* larvae was provided by the loss of bacterial morphogenic capacity on isolation from soluble algal compounds. This was interpreted as intimating that either compounds both from coralline algae and bacteria are required for metamorphic induction, or that a precursor substrate from the host alga is required for the bacteria to produce the morphogenic agent. For the purpose of both hypotheses, however, bacteria constitute an essential requisite for metamorphic induction previously attributed exclusively to the host algae.

### Morphogenic Properties of Juvenile and Adult Prey

Carnivorous invertebrates are frequently found to settle and metamorphose in close association with their prey (see reviews of Scheltema, 1974; Pawlik, 1992; Rodriguez *et al.*, 1993). Examples have been documented among the Opisthobranchia (see review by Havenhand, 1991), Holothurioidea, Asteroidea and Zoantharia (Chia & Spalding, 1972).

Larvae vary in both the degree of specificity, and their dependence upon, exogenous morphogenic cues (Morse, 1990). Amongst the Mollusca, for example, many species metamorphose in response to highly specific cues (Hadfield, 1974). Those invertebrate species which respond to highly specific metamorphic cues have previously been regarded as requiring the close association of conspecifics or prey species in their later life history. Those species which exhibit highly specialized adult prey requirements (stenophagy) for example require an especially close association with the specific food source. The larvae of stenophagous species must therefore settle and metamorphose on or near these often patchily distributed and sessile food sources (Chia, 1978; Hadfield, 1984; Hadfield & Switzer-Dunlap, 1984; Hadfield & Pennington, 1990; Pawlik, 1992).

Many opisthobranch molluscs in particular tend to exhibit stenophagy (Hadfield, 1984). Thus dorid nudibranchs predominantly prey on specific sponge species and bryozoans whilst aeolid nudibranchs tend to prey on cnidarians (Todd, 1981, 1985; Faulkner & Ghiselin, 1983; Hadfield & Miller, 1987). Nudibranchs frequently require the presence of the specific adult prey item to initiate metamorphosis (Thompson, 1958a; Perron & Turner, 1977; Chia & Koss, 1978; Hadfield, 1978a; Rodriguez *et al.*, 1993). Both the close predator-prey association between many nudibranchs and their adult food source and their highly specific morphogenic cue requirements are demonstrated by Table 4.2. and will be fully discussed in Section 4.4.

### The Nature of Nudibranch Metamorphic Cues

To date, information concerning the chemical identity of marine invertebrate morphogenic cues is scarce (see Pawlik, 1992). This trend is reflected within the Order Nudibranchia, which will be discussed here. Whilst the first isolation and identification of a nudibranch metamorphic cue remains to be made, more extensive

**Table 4.2. Nudibranch species and the biogenic sources of their metamorphic cue substances.** The reliance of many nudibranchs on the presence of the primary prey species initiation of metamorphosis is readily apparent. —

Species	Source of Metamorphic Cue	Association	Nature of Action	Authoritory
<i>Doridella obscura</i>	<i>Electra crustulenta</i>	Adult Prey	Contact	Perron & Turner (1977)
<i>Eubranchus doriae</i>	<i>Kirchenpaueria pinnata</i>	Adult Prey	Non-contact	Bahamondes-Rojas (1988), Bahamondes-Rojas & Dherbomez (1990)
<i>Berghia verrucicornis</i>	<i>Aiptasia pallida</i>	Adult prey	Non-contact	Carrol & Kempf (1990)
<i>Tritonia hombergi</i>	<i>Alcyonium digitatum</i>	Adult Prey		Thompson (1962)
<i>Trinchesia aurantia</i>	<i>Tubularia indivisa</i>	Unknown		Swennen (1961)
<i>Rostanga pulchra</i>	<i>Ophlitaspongia pennata</i>	Adult Prey	Contact	Chia & Koss (1978)
<i>Hypselodoris infucata</i>	<i>Dysidea</i> sp. <i>Halichondria coerulea</i> <i>Sigmodocia</i> sp. <i>Tedania macrodactyla</i>	Adult Prey Non-prey Non-prey Non-prey	Not Stated	Hubbard (1988)
<i>Onchidoris billemellata</i>	<i>Chthamalus dalli</i>	Adult Prey	Contact	Chia & Koss (1988) Todd (1979)
<i>Phestilla sibogae</i>	<i>Porites compressa</i> <i>P. lobata</i>	Adult Prey	Non-contact	Hirata & Hadfield (1986) Hadfield & Scheur (1985) Hadfield & Pennington (1990)
<i>Eubranchus farrani</i>	<i>Obelia geniculata</i>	Possible juvenile diet	Non-contact	Todd (1981)
<i>Onchidoris muricata</i>	<i>Electra pilosa</i>	Adult Prey		Todd & Havenhand (1985)
<i>Archidoris pseudoargus</i>	<i>Halichondria panicea</i>	Adult Prey	Contact	*Todd & Havenhand (1985)
<i>Adalaria proxima</i>	<i>Electra pilosa</i>	Adult Prey	Non-contact Contact Non-contact	Thompson (1958) Todd <i>et al.</i> (1991) Lambert & Todd (1994)

\* but see Todd (1987).



information is available regarding the nature of action of these unknown substances.

It is apparent from Table 4.2 that the nature of metamorphic cues is not uniform. The morphogenic effect of some cues is dependent upon larval contact with the biogenic substratum. Such is the case for *Doridella obscura* (Perron & Turner, 1977) and *Onchidoris bilamellata* (Todd, 1979; Chia & Koss, 1988). Conversely, other nudibranchs may be induced to metamorphose by cues present within the water column. Examples include *Berghia verrucicornis* (Caroll & Kempf, 1990), *Eubbranchus doriae* (Bahamondes-Rojas, 1988), *Phestilla sibogae* (Hirata & Hadfield, 1986), *Eubbranchus farrani* (Todd, 1981) and *Adalaria proxima* (Thompson, 1958).

To date there have been two partial purifications of nudibranch metamorphic cues -for *Eubbranchus doriae* and *Phestilla sibogae*. *E. doriae* metamorphoses in response to an aqueous extract of the adult prey hydroid *Kirchenpaueria pinnata* (Bahamondes-Rojas, 1988; see Table 4.2). More recent investigations revealed the morphogenic cue to be a polar (water soluble) molecule of approximately 1000 Da containing galactosidic residues (Bahamondes-Rojas & Dherbomez, 1990). The authors successfully induced metamorphosis in this nudibranch using purified solutions of hexoses and galactosamine with stereo-specific (cis) hydroxyl groups and proposed these sugar radicals to be acting by mimicking the reactive element of the natural metamorphic cue molecule.

Extensive research has concerned the association between *Phestilla sibogae* and its adult prey, the stony corals *Porites compressa* and *P. lobata* (Hadfield, 1977; 1984; Hadfield & Scheuer, 1985; Hirata & Hadfield, 1986; Hadfield & Pennington, 1990). It was Harris (1973) who first documented that the presence of *P. compressa* had the capacity to induce metamorphosis in *P. sibogae* veliger larvae without contact. This naturally induced metamorphosis was subsequently established to be mediated by a mechanism distinct from artificial (choline-induced) metamorphosis by Hirata & Hadfield (1986) who showed that the natural metamorphic cue bound to different receptor sites than those bound by choline. Hadfield and Scheuer (1985) found the cue molecule to be relatively thermally (0-100°C) and pH (1-10) stable. The maximum metamorphic response was evident 28-32 h after initial exposure and dilution rendered the cue ineffective 2-3 cm from the coral source. Partial purification of the cue by ultra-filtration and high performance liquid chromatography (HPLC) confirmed the molecule to be small

(<500 Da) polar and water soluble with an estimated potency of  $10^{-10}$  M or less (Hadfield & Pennington, 1990). Although this is perhaps the most extensively researched of nudibranch metamorphic cues to date, it still remains to be isolated and identified.

The subject of the present study, *Adalaria proxima*, metamorphoses in response to the primary adult prey item, the bryozoan *Electra pilosa* (Thompson, 1958a & b; Todd & Havenhand, 1985; Todd *et al.*, 1991; Lambert & Todd, 1994). It was Thompson (1958a) who first addressed the larval biology and ecology of this dorid nudibranch. In his comprehensive treatise on *A. proxima* he stated that whilst both the 'smell' and 'texture' of *A. proxima* were prerequisites for the induction of settlement and metamorphosis, the 'smell' was of primary importance. The predominant influence of smell over texture was evidenced by the failure of dead *E. pilosa* to induce metamorphosis. In addition, the successful induction of metamorphosis by larvae denied direct contact with *E. pilosa* through separation with fine 'plankton silk' offered further support that experience of the texture of *E. pilosa* was not essential for the induction of metamorphosis. Further investigation performed over thirty years later by Todd *et al.* (1991) documented the results of preliminary experiments into the nature of the natural cue. In addition to live *E. pilosa* colonies, the aqueous extract and centrifuged particulates of homogenised *E. pilosa* were assayed. The failure of all treatments except that of the live *E. pilosa* colony led the authors to conclude metamorphosis to be induced by some element of the live intact bryozoan, the cue molecule being both water insoluble and unextractable in aqueous extract. Research performed subsequently by Lambert & Todd (1994) concurrent with this present study, further addressed the nature of the cue by investigating the morphogenic properties of homogenised *E. pilosa* colonies and of sea water 'conditioned' by the presence of *E. pilosa*. Utilizing the rationale of Thompson (1958a) the authors found that metamorphosis could be successfully induced in competent larvae denied contact with the live *E. pilosa* colony by fine mesh, and indeed, water conditioned by the previous presence of *E. pilosa* could likewise induce metamorphosis. These results offered support for the original findings of Thompson (1958a) over those of Todd *et al.* (1991) by providing further evidence of a water-borne metamorphic cue emanating from *E. pilosa*. The later study therefore established that larval contact with the adult prey did not constitute a pre-requisite to metamorphic induction.

## Rationale

The objective of the present study was to further elicit the nature of the natural metamorphic cue for larvae of *Adalaria proxima* emanating from the bryozoan *Electra pilosa*. This was achieved by employing a variety of separate trials aimed at characterizing distinct properties of the natural cue molecule. The primary areas of study addressed were:

### 1. Degree of Cue Specificity

Whilst it is known that *Adalaria proxima* settles and metamorphoses in response to its primary adult prey species (the bryozoan *Electra pilosa*) the degree of cue specificity displayed by *A. proxima* has not been determined. Such stenophagous predatory species frequently possess highly specific metamorphic cue requirements. The primary objective of this study therefore was to evaluate the degree of cue specificity demonstrated by *A. proxima* by exposing competent veliger larvae to range of littoral invertebrate taxa.

The bryozoan species selected for screening are within the Class Gymnolaemata (Order Ctenostomata and Cheilostomata). Cheilostome and ctenostome bryozoans may most readily be distinguished by the membranous or gelatinous zooid walls and absence of operculum in the former order in contrast to the calcified body wall and operculum typical of latter order (Hayward, 1985). Whilst *Electra pilosa* constitutes the primary food source of *A. proxima* other bryozoan species such as the cheilostome *Flustrellidra hispida* (Fabricius) and ctenostomes *Membranipora membranacea* (Linnaeus) and *Alcyonidium polyomm* (Hassall) are also fed upon (Thompson, 1958; Todd, 1981). The bryozoan species *M. membranacea*, *Fl. hispida*, *Alcyonidium gelatinosum* (Linnaeus), *Alcyonidium hirsutum* (Fleming) in addition to *E. pilosa* were therefore assayed for morphogenic properties. *M. membranacea* is found from the low shore to the sublittoral throughout the British Isles and typically is epiphytic on laminarians (Hayward, 1985; Ryland, 1990), although occasionally it occurs on *Fucus serratus* (Hayward, 1985). In contrast, *Flustrellidra hispida* may be found primarily on *Fucus serratus* (Hayward, 1985; Ryland, 1990). Unlike *E. pilosa*, *M. membranacea* and *Flustrellidra hispida*, the ctenostome Bryozoa *A. gelatinosum* and *Alcyonidium hirsutum* have no reported association with *A. proxima*. Like the other species however, these two occur either primarily on *Fucus serratus* (*A.*

*gelatinosum*) or on several macroalgal species including *Fucus serratus* (*Alcyonidium hirsutum*) (Hayward, 1985; Ryland, 1990).

In addition, members of other invertebrate taxa from the middle to low shore were screened for morphogenic activity. The Breadcrumb sponge *Halichondria panicea* (Pallas)(Class Demospongiae) is common on rocks and laminarians in the lower shore and sublittoral zone (Dyrynda & Dyrynda, 1990). The Star Ascidian *Botryllus schlosseri* (Pallas)(Class Ascidiacea) was also included. *B. schlosseri* is epiphytic on algae, ascidian tests and inert surfaces such as rock on the lower shore to down shallow water (Millar, 1970; Knight-Jones & Ryland, 1990). Colonies may be flat, encrusting or lobate (Knight-Jones & Ryland, 1990) with individual zooids forming characteristic star-shaped groups embedded within the test (Millar, 1970; Knight-Jones & Ryland, 1990).

## 2. Minimum Effective Exposure Time of the Natural Cue

Timed exposure trials were performed using the natural morphogenic cue to provide comparisons with previous timed exposure data obtained using artificial cues (choline chloride and elevated potassium) by Todd *et al.* (1991).

## 3. Relative Potency of the Natural Cue

Whilst the absolute concentration of an unknown substance cannot be determined, an appreciation of the relative potency of the cue was considered pertinent to facilitate further characterization of the cue by providing increasingly reliable bioassays and indicate the effective range of the inducer from a point source.

## 4. Other Properties

Johnson & Suttons' (1994) study of metamorphic induction in *Acanthaster planci* established the morphogenic cue -which had previously been attributed to the crustose coralline algal host- to be bacterial in origin. This provided the impetus to explore any possible association of the metamorphic cue of *Adalaria proxima* with bacteria in this present study. In addition, the thermal stability of the metamorphic cue was investigated to both provide practical information for the conservative handling of the cue during experimental manipulation, and to obtain more information concerning the structure and nature of the cue molecule.

## 4.2 METHODS

### Collection and Maintenance of Spawn Masses

All larvae utilized in this investigation were obtained from laboratory cultured parent stocks and experiments were undertaken between 1993 and 1994 during two annual spawning seasons. Adult *A. proxima* were collected from the field and both parent stock and subsequent spawn masses were maintained within the laboratory as previously described in Chapter 3 (Section 3.2). At no time were antibiotics administered during the culture of adults or spawn masses.

### Collection of Sample Organisms

*F. serratus* with epiphytic *E. pilosa* was collected from the low shore at St. Andrews and Kings Barns, Fife, and from Clachan Seil, Argyll during low spring tides. *E. pilosa* colonies were retained on their immediate section of *F. serratus*, although excess *F. serratus* was carefully trimmed away without damaging the bryozoan colonies. The resulting *E. pilosa* colonies were rinsed with clean TFSW and conditioning performed as soon as possible.

A range of organisms including *Fucus serratus* and associated epiphytic invertebrate fauna were collected at sites from which *Electra pilosa* had previously been obtained (St. Andrews and Kingsbarns, Fife, and Clachan Seil, Argyll). The previously described protocol employed for the preparation of clean *E. pilosa* specimens was applied for the ascidian *Botryllus schlosseri* and all other bryozoans.

*H. panicea* was obtained from Kingsbarns, Fife (Experiment 3) and Clachan Seil, Argyll (Experiment 5). Whilst this sponge may occur in an encrusting or massive form, only small specimens of the massive form were utilized for this study to ensure the sponge remained as undisturbed as possible between collection and experimental use. After collection, the cut sponge was placed in flowing seawater within the laboratory for at least 24 h to improve survival during the



subsequent bioassay (see Hubbard 1988). Colonies of the ascidian *Botryllus schlosseri* may be flat, encrusting or lobate on rock or algae. This study utilized only colonies encrusting *F. serratus* collected from Clachan Seil, Argyll (Experiments 4 & 5).

#### Preparation of Artificial Cues

Veliger larvae were subjected to a choline ( $5 \cdot 10^{-3} \text{M}$ ) positive control treatment in every experiment to confirm competency. This solution was prepared immediately prior to initiation of each experiment using twice filtered ( $0.45 \mu \text{ min}$ , thence  $0.2 \mu \text{ min}$ ) sea water hereafter referred to as TFSW. The morphogenic properties of elevated potassium ion concentration on *Adalaria proxima* have been previously addressed in Chapter 3. Whilst *A. proxima* larvae have been established to be sensitive to a rise of just 2.5mM above usual sea water potassium ion concentration, Todd *et al.* (1991) established the optimal metamorphic response to be elicited by 19mM, which is 10mM above the usual sea water potassium ion concentration. Elevated potassium ion concentration (19mM) is regarded therefore as a reliable indicator of the attainment of metamorphic competence in *A. proxima* (Todd *et al.* 1991). Elevated potassium ion concentration was used as a positive control only once in this series of experiments, in Experiment 8. This was obtained by simple supplementation of TFSW with potassium chloride (Sigma Chemicals) to produce a final potassium ion concentration of 19mM.

#### Preparation of Sea Water Conditioned using *Electra pilosa*

The natural cue was provided by either subjection of the larvae to live colonies of the bryozoan *E. pilosa* (Experiment 3), or to water 'conditioned' by previous exposure to *E. pilosa* (all other experiments). The conditioning protocol has been previously documented in Chapter 3 (section 3.2). The absolute concentration of the unknown cue obviously could not be determined and therefore a standard of 40 g (damp wt.) of *F. serratus* and *E. pilosa* to 500 ml TFSW was used to condition water. Whilst it is apparent that the conditioning properties of *E. pilosa* may not have been constant, standard conditioning did at least exclude one source of variability and provided a degree of inter-experiment comparison.



A protocol for the reliable production of 'active' conditioned sea water was required to facilitate investigation of the properties of the cue. The variable morphogenic efficacy of the natural cue apparent during the course of this study, also noted by Todd & Lambert (1994), therefore necessitated the investigation of optimal conditions required for the successful conditioning of water by *E. pilosa*. The results for a number of experiments were discarded during the initial stages of the study due to the complete failure of *E. pilosa* conditioned water to effect metamorphosis among competent larvae. Possible factors contributing to a reduction, or indeed absence, of cue activity were therefore considered. Conditioning of water at 6°C proved consistently unsuccessful. Colonies incubated at a range of temperatures (6°C, 10°C, 13°C and 22°C) were therefore examined under a binocular microscope. Whilst lophophore activity and a retractive response were apparent under all temperature regimes except 6°C.

All those trials in which 'active' sea water was obtained utilized colonies collected within the preceding 5 d. Whilst a range of *E. pilosa* colony sizes were available at all sites from which collection took place, preliminary trials showed the smaller (and by inference younger) colonies of approximately 2-4 cm length and 1-3 cm width to be effective in producing 'active' conditioned water. Trials in which polystyrene cell-wells were used as treatment dishes were not successful, and for this reason glassware was subsequently utilized in all experiments during both the conditioning of sea water and bioassays. Active conditioned water was obtained after 1-3 d exposure to *E. pilosa*. Concurrent work by Dr. W. Lambert confirmed many of these factors and also established a high surface area to volume ratio of sea water, agitation and/or aeration to be prerequisites of effective conditioning.

In summary, preliminary trials confirmed the successful conditioning of sea water to be facilitated by freshly collected young colonies of *E. pilosa* retained upon *F. serratus* and incubated in an aerated glass container at 10°C for 1-2 days. Consequently the conditioning of sea water by *E. pilosa* was performed in this manner for all experiments presented hereafter.

#### General Experimental Protocol

Samples were divided into 3-5 replicates per treatment with a minimum of 10 larvae per replicate. Larvae were maintained within autoclaved (115°C for 15min) glass petri dishes (diameter 55mm, height 20mm) for the duration of each

experiment with 15-20ml of treatment solution. Where small spawn mass size precluded the use of sibling veligers within each separate experiment, progeny of the same age from several spawn masses were mixed before division into treatments and replicates. Each experiment was monitored using a Wild-E binocular microscope on Day +3 after initiation of the experiment and thereafter on Day +6 unless the positive control had previously elicited a maximal response or high mortality precluded the second observation. Daily observation was not performed as the primary objective of this study was to determine the morphogenic capacity of treatments from the absolute metamorphic responses elicited, not the relative rates of response. Larval status was recorded, in the same manner as was applied for Chapter 3, either as pediveliger, spontaneous evacuee, successful metamorph or dead. All experiments were undertaken at 10°C.

This general protocol was employed for all experiments in this chapter. The more specific protocols required to examine aspects of the natural cue are detailed below.

#### Cue Specificity (Experiments 1 - 5)

Upon hatching veliger larvae move into the water column, leaving an empty spawn mass behind. It was this egg mass jelly, or stroma, that was used to provide material for the *A. proxima* stroma treatments (Experiments 1 - 3).

In Experiment 3 the species under examination were placed in the treatment dishes for the duration of the experiment, thus allowing larvae contact with the substratum. This caused a high concentration of organics in the water and resulted in raised mortality of larvae and visible deterioration in the health of the test species. Consequently, subsequent trials (Experiments 4 & 5) used water conditioned by the species under investigation. The conditioning process was performed in an identical fashion to that employed in the successful production of *E. pilosa* conditioned water.

#### Cue Exposure Experiments (6 - 7)

Veliger larvae were immersed in conditioned sea water for a timed interval ranging from 10s to continuous exposure. Competence of larvae was confirmed by

inclusion of a  $5 \cdot 10^{-3} \text{M}$  choline positive control treatment. Manipulation of veligers was performed by pipetting under a binocular microscope. On removal from the CSW treatment larvae were twice rinsed by immersion in twice filtered sea water (TFSW) and transferred to glass petri dishes for incubation at  $10^{\circ}\text{C}$  and subsequent monitoring.

#### Cue Potency Experiments (8 - 12)

Serial dilution of CSW was from full strength (100%) down to 0.0001% of its initial concentration. Solutions were thoroughly agitated between each dilution. Larvae were transferred to each treatment in order of decreasing dilution factor to minimize the danger of contamination by a less diluted treatment. Fresh autoclaved glass pipettes were used for each treatment. Whilst 3 replicates per treatment were initially employed (Experiments 8 & 9) the observed degree of between replicate variation in response resulted in the number of replicates being subsequently increased to five (Experiments 10 - 12).

#### Experiments Investigating Other properties of the Natural Cue

All sea water used in controls and for the conditioning of sea water was filtered using a  $0.45\mu$  min filter. For those experiments (4, 9, 10, 12 & 13) in which filtering constituted a treatment, *E. pilosa* CSW was filtered after conditioning and immediately before commencement of the experiment using a sterile disposable  $0.2\mu$  min Nalgene® Syringe filter (25mm cellulose acetate membrane).

Investigations into any microbial association with the cue required the administration of antibiotics as treatments within several experiments (1, 13 & 14). The antibiotic mix administered was composed  $43.7 \text{ mg l}^{-1}$  Streptomycin sulphate (Sigma Chemical Co.) and  $52.5 \text{ mg l}^{-1}$  Penicillin-G (Benzylpenicillin, Sigma Chemical Co.) in TFSW.

*E. pilosa* skeletons utilized in Experiment 15 were obtained by the desiccation of colonies grown upon coverslips. Coverslips were attached to microscope slides for increased robustness and conditioned by placing in TFSW either with *F. serratus* only (control treatment) or with *E. pilosa* epiphytic on *F. serratus*. The resultant filmed coverslips then were gently rinsed in TFSW,

removed from the microscope slides, and placed in the bioassay dishes in TFSW. The *E. pilosa* conditioned water was bioassayed as a separate treatment to confirm activity.

In order to obtain a bacterial film (Experiment 14), associated with conditioned sea water, autoclaved glass petri dishes were immersed in sea water which then was conditioned by the addition of *E. pilosa*. The dishes were removed after 3 days, emptied, rinsed in TFSW at ambient and filled with fresh unconditioned TFSW. One treatment was then left untreated, whilst antibiotics were administered to the other. Active conditioning of the sea water was confirmed by the inclusion of a CSW treatment in an unfilmed petri dish.

Investigation into the thermal stability of the cue molecule required conditioned water to be frozen at -20°C for between 12h (Experiment 5, 10 - 12) and 11d (Experiment 12). Frozen samples were defrosted at ambient (10°C) before use. Previously boiled CSW was used in Experiment 12. CSW held within a Pyrex flask was heated to 100°C using a Bunsen burner. The sample was then left to cool until reaching room temperature and subsequently lowered to 10°C by placing in an incubator.

#### Lipid Extraction of Conditioned Sea Water

Investigation of the chemical nature of the natural cue molecule was performed by the partitioning of CSW into its constituent aqueous and organic fractions using a modified Folch method (Christie 1982). Sub-samples (500ml) of *F. serratus* & *E. pilosa* CSW obtained using the standard protocol previously documented in this chapter were prepared for extraction by filtering through glass wool in order to remove particulates. Aliquots of these primary samples were bioassayed prior to partitioning in order to confirm the morphogenic activity of the CSW (and therefore by inference the presence of the cue molecule).

Lipid extraction was performed by the addition of 30ml chloroform : methanol solution (2:1 v/v). After agitation the organic (lower) fraction was decanted from the main. This procedure was repeated 3 times. The resulting organic fraction (90ml total) then was washed by the addition of 1% potassium chloride solution in TFSW (Experiment 15 only) and the aqueous fraction removed. This procedure was repeated twice.

The organic fraction then was dried to eliminate solvents using a GeneVac SF 50 rotary evaporator to obtain the purified lipid sample. Resuspension of purified organic samples was achieved by addition of TFSW (organic fraction) or distilled water (aqueous fraction) and sonication at 3 $\mu$ m amplitude in a Soniprep 150 sonicator. Sea water conditioned exclusively with *F. serratus* was utilized throughout as a negative control. All bioassay of resuspended fractions was performed using the same protocol as previously documented in this chapter.

### Data Analysis

Data were analysed following the procedures previously detailed in Chapter 3 (Section 3.2). Data are presented in Tables and Figures as back-transformed angular means (in percentage terms), and, where appropriate, are displayed with associated standard error bars.

## 4.3 RESULTS

### Cue Specificity

Table 4.3 summarizes the results of five experiments designed to assess the degree of specificity displayed by the natural metamorphic cue of *A. proxima*. The competence of veligers was confirmed by inclusion of  $5 \cdot 10^{-3}$ M choline chloride controls throughout. Treatments in Experiment 1 were performed in triplicate. As a consequence of the degree of within-treatment variation observed in metamorphic response, all treatments (Experiments 2 - 5) were thereafter quintuplicated.

### The Morphogenic Properties of *A. proxima* Egg Mass Jelly (Stroma)

Experiments (1 - 3) investigated the morphogenic properties of *A. proxima* stroma. In no experiment in which competent veliger larvae were exposed to stroma were significant ( $P < 0.05$ ) metamorphic responses elicited (see Figures 4.1 a, b & c).

### The Morphogenic Properties of Bryozoa (Class Gymnolaemata)

Five species of cheilostome and ctenostome bryozoa, all of which may be found encrusting *F. serratus*, were screened for morphogenic capacity with respect to *A. proxima*. The back-transformed mean metamorphic responses are presented in Table 4.3 (Experiments 3 - 5). Significant levels of metamorphosis (98.3%, 58.2% and 99.6% respectively) were elicited by  $5 \cdot 10^{-3}$ M choline in each experiment, confirming that the larvae were competent.

It is notable that *E. pilosa* was the only bryozoan species found to elicit a significant metamorphic response among *A. proxima* veliger larvae (see Table 4.3). Whilst between-experiment variation in metamorphic response (40.3%, 25.2% and 20.9% in Experiments 3, 4 & 5 respectively) is evident, significant levels of metamorphosis were induced in all experiments. The results of multiple comparisons performed by Tukey's HSD and presented above the relevant



**Table 4.3. Results of screening a range of taxa for morphogenic capacity with respect to competent veliger larvae of *Adalaria proxima*.** Data are back-transformed mean metamorphic responses for each quintuplicate treatment (except Experiment 1 which was triplicated) expressed as percentage responses. Dashes (-) indicate those treatments for which no data are available. Integers in parenthesis denote the number of separate experiments composing the mean. Multiple comparisons within each experiment are illustrated graphically by Figures 4.1a - e.

Treatment	Experiment					Mean Treatment Response
	1	2	3	4	5	
Sea Water	0	0	0	0	0.4	0.1 (5)
5mM Choline	86.9	64.5	98.3	58.2	99.6	81.5 (5)
<i>Adalaria proxima</i> stroma	1.1	3.7	0.4	-	-	1.7 (3)
Class Gymnolaemata						
Order Cheilostomata						
<i>Electra pilosa</i>	-	-	40.3	25.2	20.9	28.8 (3)
<i>Membranipora     membranacea</i>	-	-	-	-	0	0 (1)
Order Ctenostomata						
<i>Flustrellidra hispida</i>	-	-	3.4	1.7	9.6	7.3 (3)
<i>Alcyonidium gelatinosum</i>	-	-	8.0	0	2.4	3.5 (3)
<i>Alcyonidium hirsutum</i>	-	-	0	-	0	0 (2)
Class Ascidiacea						
<i>Botryllus schlosseri</i>	-	-	-	0.4	0	0.2 (2)
Class Demospongiae						
<i>Halichondria panicea</i>	-	-	0.4	-	25.2	12.8 (2)
Class Phaeophyceae						
<i>Fucus serratus</i>	-	-	1.7	-	-	1.7 (1)

responses in Figures 4.1c - e illustrate that whilst the metamorphic responses to *E. pilosa* are significant, in each case they are lower than those elicited by  $5 \cdot 10^{-3} \text{M}$  choline.

The ctenostome *F. hispida* triggered 3.4%, 1.7% and 9.6% metamorphosis in Experiments 3, 4 & 5 respectively. One way ANOVA and subsequent Tukey's HSD established none of these responses to be significantly greater than that of the sea water controls (see Figures 4.1 c-e). The 9.6% metamorphosis triggered by *F. hispida* in Experiment 5 (see Figure 4.1e) does however represent the sole example in this investigation of a bryozoan-induced metamorphic response not significantly lower than that elicited upon treatment with *E. pilosa*.

#### The Morphogenic Properties of Other Taxa

Neither the ascidian *B. schlosseri* (see Figures 4.1 d & e) or the fucoid *F. serratus* (see Figure 4.1 c.) displayed significant morphogenic properties. The sponge *H. panicea* was included in both Experiments 3 & 5. Table 4.3 shows that whilst only 0.4% of larvae underwent metamorphosis in response to *H. panicea* in Experiment 3, this figure increased to a significant 25.2% in Experiment 5. The back-transformed mean metamorphic responses recorded in Experiment 5 are illustrated in Figure 4.1 e. This response lies within the same Tukey group as that of *E. pilosa*.

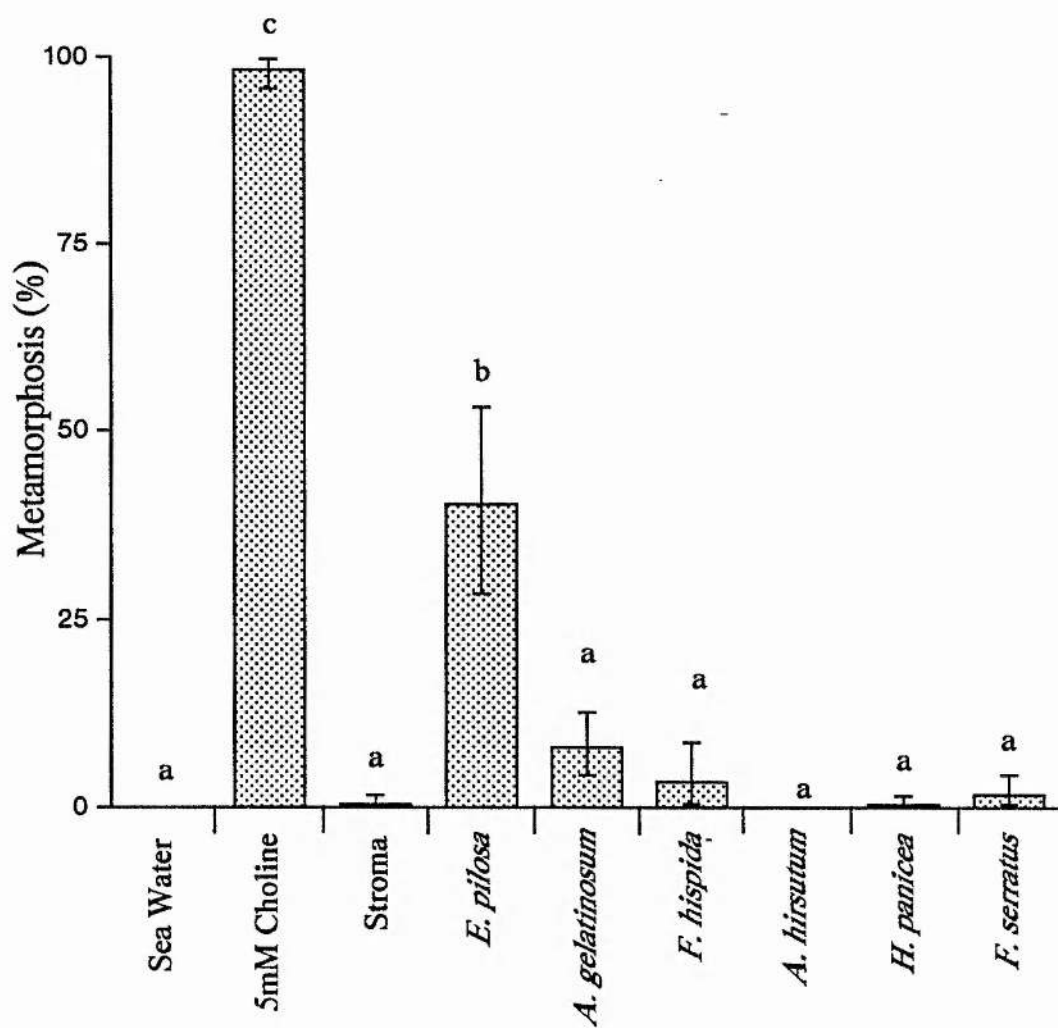
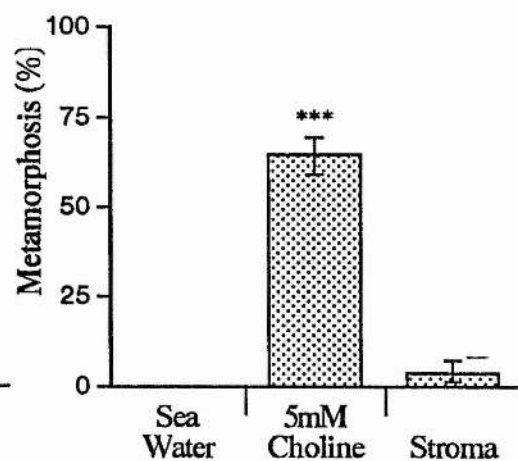
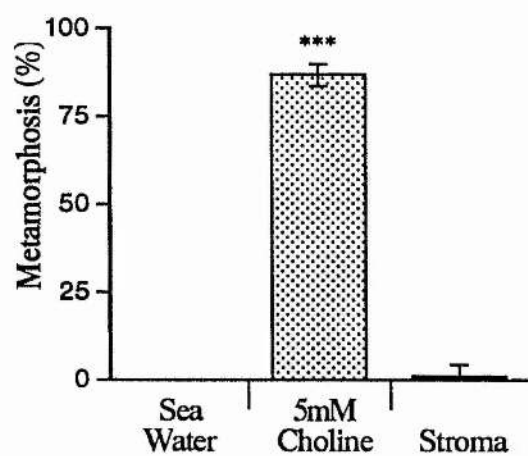
#### Cue Exposure

The back-transformed mean metamorphic responses obtained by timed exposure trials of the veliger larvae of *A. proxima* to the natural cue are presented in Table 4.6. In both Experiments 6 & 7 significant levels of metamorphosis in response to  $5 \cdot 10^{-3} \text{M}$  choline treatments confirmed the larvae to be competent. The results of Experiment 6 & 7 are illustrated with appropriate Tukey groupings in Figures 4.2 a & b respectively.

Experiment 6 was comprised of time periods ranging from continuous exposure down to 2 min. A oneway ANOVA established a significant between-treatment differences in metamorphic response ( $P < 0.001$ ), and subsequent multiple

**Figures 4.1 a & b** (above left and above right respectively). The metamorphic induction capacity of *A. proxima* egg mass jelly (stroma) on competent veliger larvae. Data are back-transformed means and associated standard errors. Asterisks denote those responses considered significantly different to the sea water controls at the  $P < 0.05$  level.

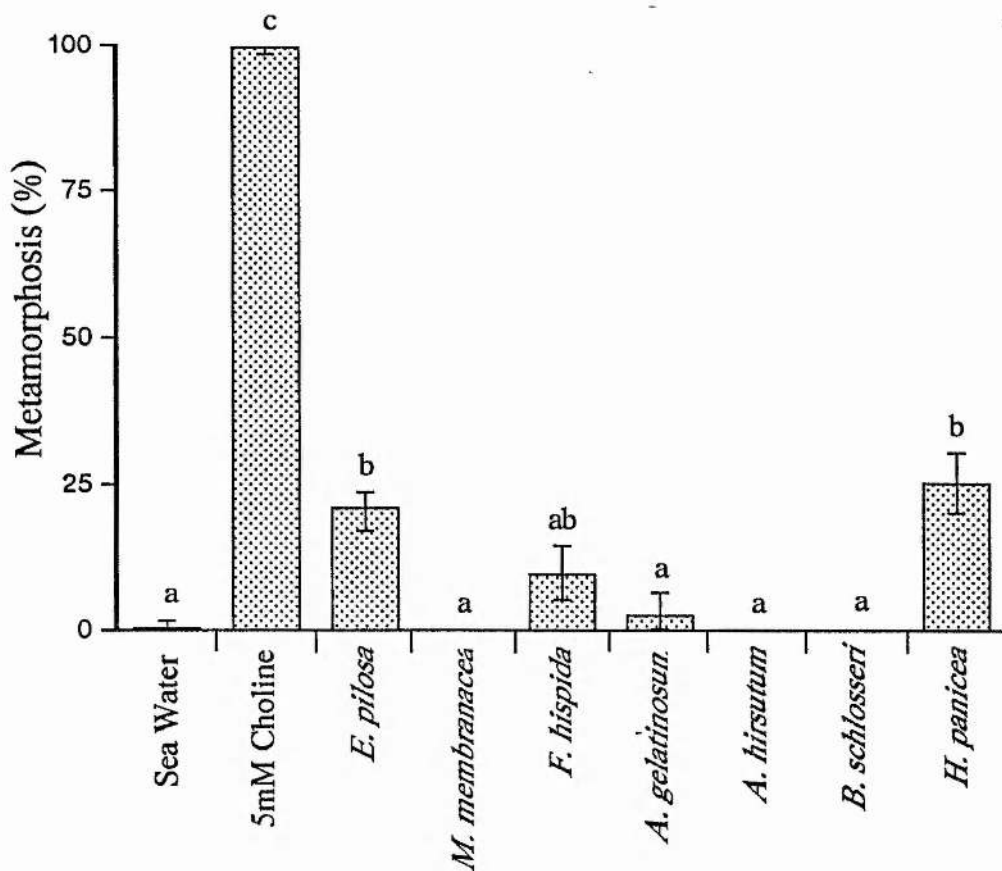
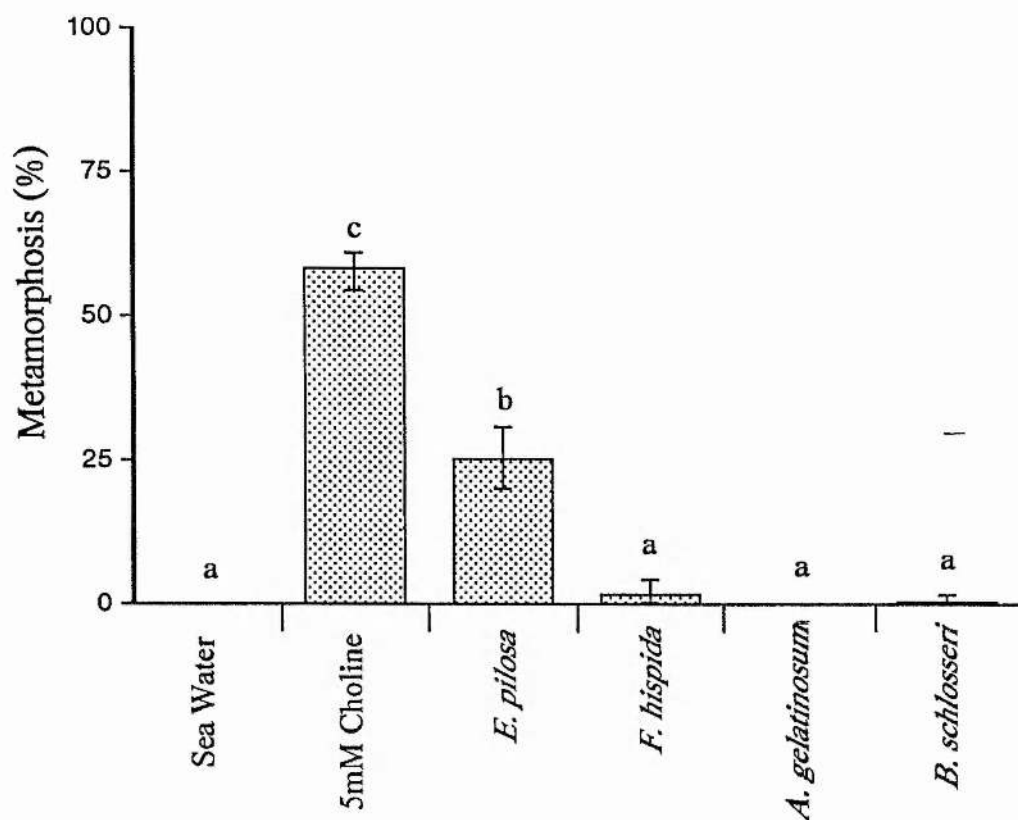
**Figure 4.1 c** (below right). Experiment 3. Induction of metamorphosis in larvae of *A. proxima* by a range of lower shore organisms. Data are back-transformed means and associated standard errors ( $n=5$  replicates per treatment). Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P < 0.05$  significance level. Only the  $5 \cdot 10^{-3}M$  choline and *E. pilosa* treatments were proved to elicit significant levels of metamorphosis.



**Figures 4.1 d (above right). Experiment 4. Induction of metamorphosis in larvae of *A. proxima* by both cheilostome and ctenostome bryozoans and an ascidian.** Data are back-transformed means and associated standard errors (n=5 replicates per treatment). Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P < 0.05$  significance level. Only the 5mM choline and *E. pilosa* treatments were proved to induce significant levels of metamorphosis.

**Figures 4.1 e (below right). Experiment 5. Induction of metamorphosis in larvae of *A. proxima* by members of the Bryozoa, Ascidiacea, and Demospongiae.** Data are back-transformed means and associated standard errors (n=5 replicates per treatment). Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P < 0.05$  significance level. In addition to the 5mM choline and *E. pilosa*, the *H. panicea* treatment also elicits a significant metamorphic response.





**Table 4.4.** Exposure times required for the induction of metamorphosis in *A. proxima* by *E. pilosa* conditioned sea water (CSW). Data are back-transformed mean metamorphic responses for each triplicate treatment expressed as percentage responses. Figure are maximum metamorphic responses obtained on termination of the experiments (Day +6). Dashes (-) indicate those treatments for which no data are available. Multiple comparisons within each experiment are illustrated graphically by Figures 4.2 a & b.

Treatment		Experiment	
		6	7
Sea Water		0	16.4
5mM Choline		98.8	84.2
CSW Timed Exposure			
	Continuous	23.2	88.4
	2h	22.5	-
	1h	19.4	53.3
	30min	23.2	46.7
	10min	13.0	47.0
	2min	15.8	36.6
	30s	-	19.8
	10s	-	2.4

comparisons by Tukey's HSD revealed choline to elicit the greatest response (98.8%). All CSW treatment responses down to and including 2 min exposure were significantly greater than those elicited by sea water, and Figure 4.2 a. illustrates the absence of difference in metamorphic response between exposure times at the  $P < 0.05$  level.

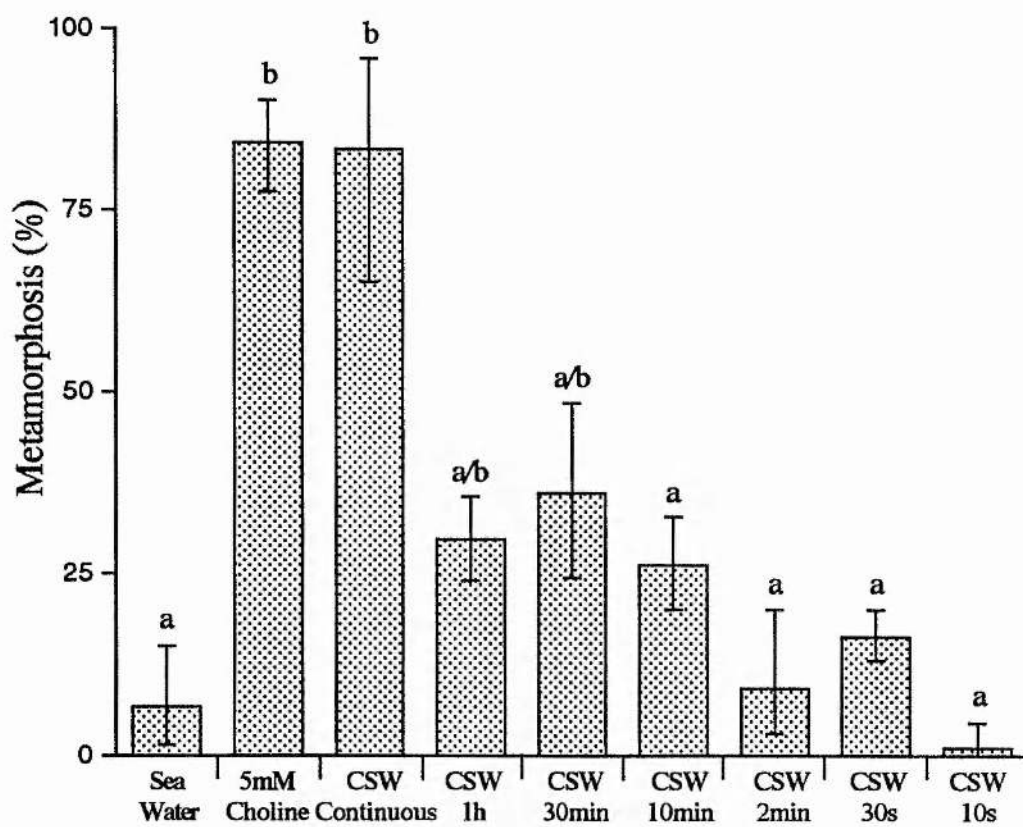
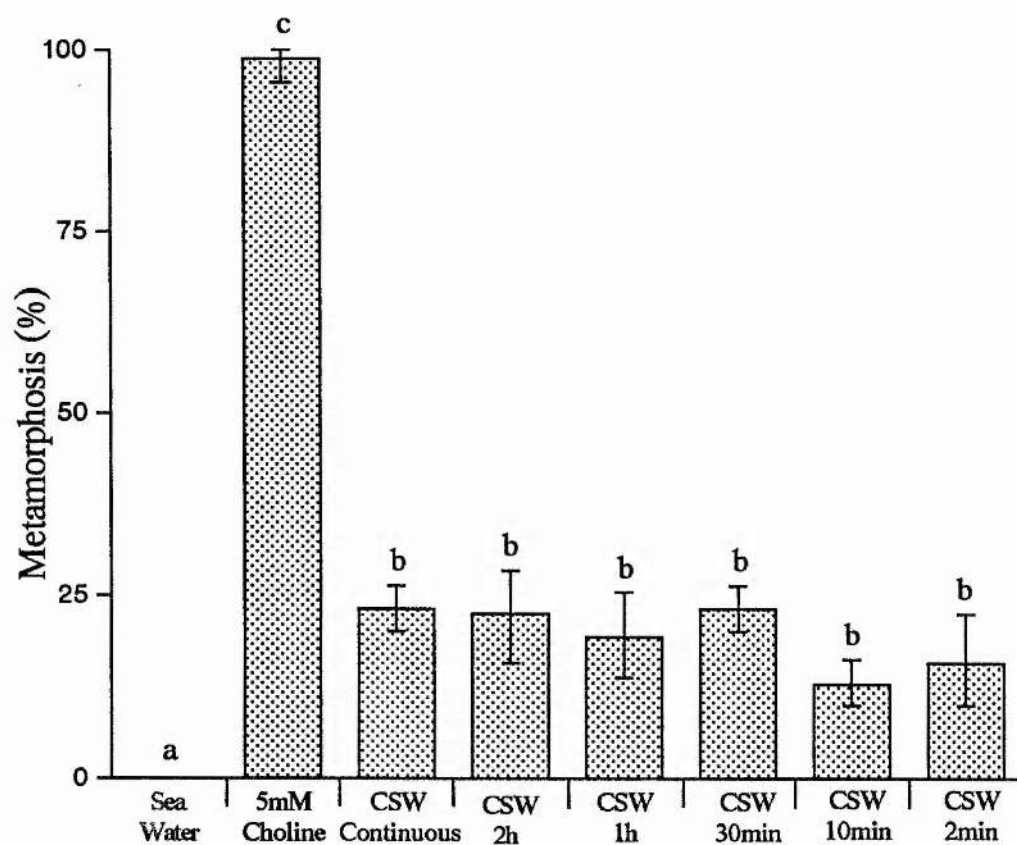
As a consequence of the significant response elicited by the minimum exposure time treatment (2min) included in Experiment 6, lesser exposure times of 30s and 10s were incorporated into Experiment 7. The time course for Experiment 7 is illustrated in Figure 4.2 c. By Day +3 the only treatments to induce significant metamorphic responses were choline (84.2%) and continuous exposure to CSW (83.3%). In contrast with the results of Experiment 6, the remainder of the exposure treatments (10s, 30s, 2min, 10min, 30min and 1h) did not trigger significant levels of metamorphosis. The two longer exposure periods of 30min and 1h triggered 36.1% and 29.7% metamorphosis respectively, and multiple comparisons by Tukey's HSD showed that these responses were not significantly lower than those of the continuous exposure treatment (see Fig 4.2 b). By the termination of the experiment at Day +6, the maximum levels of metamorphosis continued to be in response to both choline (84.2%) and continuous exposure to CSW (88.4%). The metamorphic response elicited by the continuous exposure treatment was not considered significantly higher than the 1h (53.3%), 10m (47.0%) or 30m (46.7%) exposure treatments.

#### Potency Of The Metamorphic Cue

Experiments 8 - 9 examine the potency of conditioned water (and by inference the natural cue substance) by investigating the effects of serial dilution on the morphogenic capacity of the cue. The increase in replicate number from triplicated (Experiments 8 - 10) to quintuplicated (Experiments 11 & 12) was a result of the degree of variability observed in previous experiments. The relevant back-transformed mean metamorphic responses are presented in Table 4.5. Significant metamorphic responses to the 19mM potassium control in Experiment 8 and  $5 \cdot 10^{-3}$ M choline control in all later experiments (9 - 12) confirmed the competent status of the larvae.

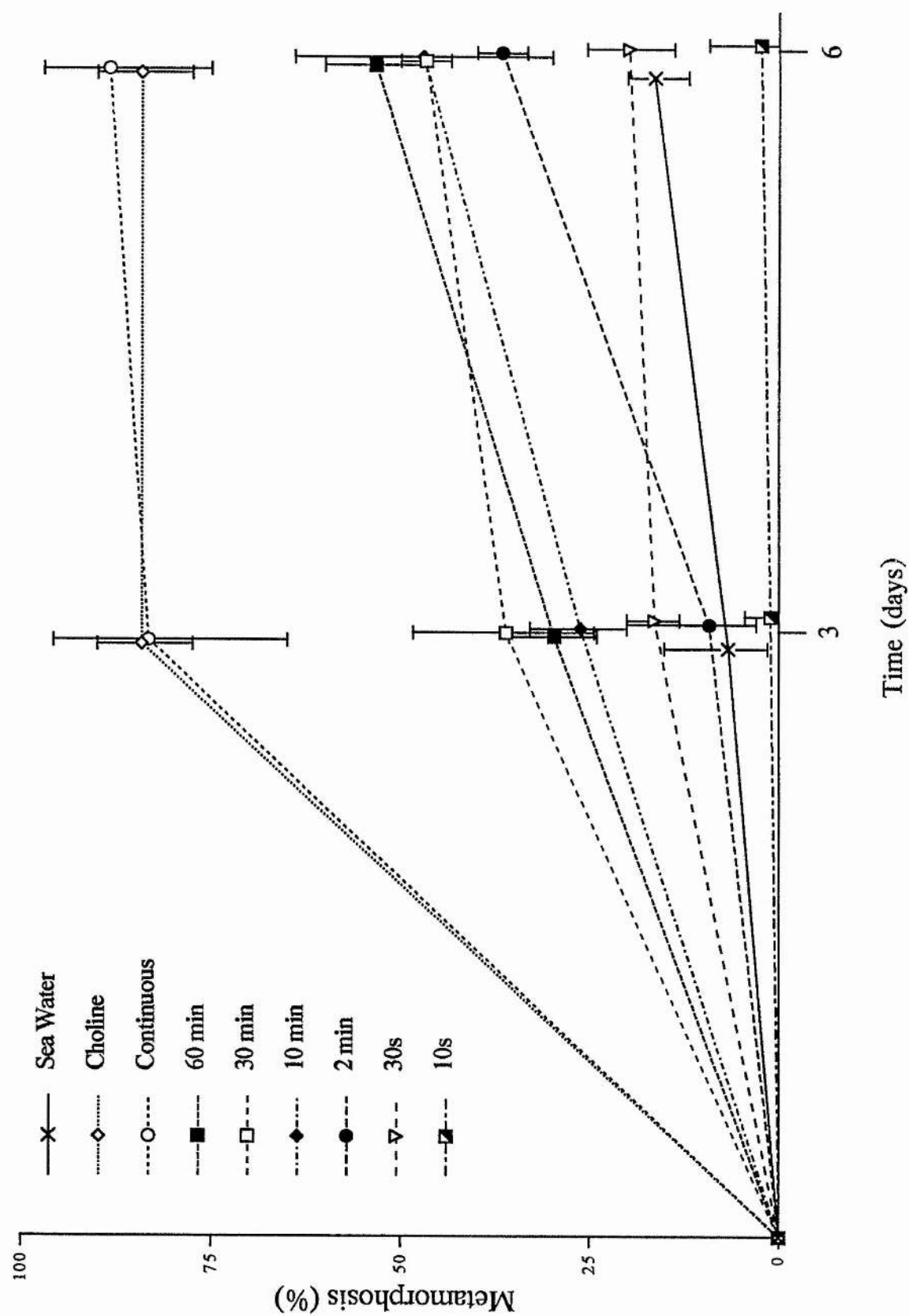
**Figure 4.2 a (above right). Experiment 6. Metamorphic response of *A. proxima* veliger larvae on exposure to *E. pilosa* conditioned sea water (CSW) for between 2min and continuous timed periods. Data are back-transformed means and associated standard errors (n=3 replicates per treatment). Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P<0.05$  significance level.**

**Figure 4.2 b (below right). Experiment 7. Metamorphic response of *A. proxima* veliger larvae exposed to *E. pilosa* conditioned sea water (CSW) for between 10s and continuous timed periods. Data are back-transformed means and associated standard errors (n=3 replicates per treatment). Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P<0.05$  significance level.**





**Figure 4.2 c. Experiment 7. Time course of metamorphic response on exposure to *E. pilosa* conditioned sea water (CSW).** Data are the larval metamorphic responses on Days +3 and +6 after initiation of the experiment. Points are back-transformed means with associated standard error bars (n=3 replicates per treatment). For absolute values of back-transformed means see Table 4.6.



*E. pilosa* CSW treatments of 100%, 50%, 10% and 1% of original CSW concentration all triggered significant metamorphic responses in Experiment 8. Whilst the maximum level of metamorphosis (94.2%) was elicited by 100% CSW, subsequent multiple comparison by Tukey's HSD established there to be no significant difference in the level of metamorphosis induced by any of these treatments (see Figure 4.3 a.). With a metamorphic response of 25.8%, 0.1% CSW (see row [L] Table 4.5) was the only treatment to fail to induce any significant level of metamorphosis within this experiment, although it was not considered significantly lower ( $P < 0.05$ ) than that of the potassium control.

A greater range of treatments, encompassing 100% CSW down to 0.01% CSW dilution, were bioassayed in Experiment 9 (see Table 4.5). Although the highest response of 89.5% metamorphosis was triggered by 50% CSW (see Table 4.5, row E), Tukey's HSD established this not to be significantly greater than that induced by either choline (66.7%), undiluted CSW (53.8%) or 20% CSW (59.2%). The most diluted treatment to trigger a significant metamorphic response was that of 1% CSW (Table 4.5, row [L]). In addition to the 0.1% and 0.001% CSW treatments the 5% CSW treatment failed to trigger a significant response (16.9%). This was unexpected given the significant response triggered by the 1% treatment, although Figure 4.3b does show there to be no significant difference between the 10%, 5%, 1%, 0.1% or 0.01% CSW metamorphic responses ( $P < 0.05$ ).

The CSW assayed in Experiment 10 showed a reduced potency relative to previous experiments (8 & 9) although an identical conditioning protocol was applied. Not only was the maximum response induced by 100% CSW only 40.8% (see Table 4.5, row [D]) but the minimum CSW concentration to produce a significant metamorphic response was 20% CSW (see Figure 4.3 c.). Whilst neither 10%, 1% or 0.01% CSW produced metamorphic responses significantly higher than that of the sea water control, the results of Tukey's HSD displayed in Figure 4.3 c. show these responses to be not significantly lower than the undiluted, 50% and 20% CSW treatments. Indeed, even the response of 2.1% (Table 4.5, row [N]) displayed by the most diluted treatment of 0.001% CSW is not significantly different to the response of 27.8% (Table 4.5, row [G]) triggered by the 20% CSW treatment.

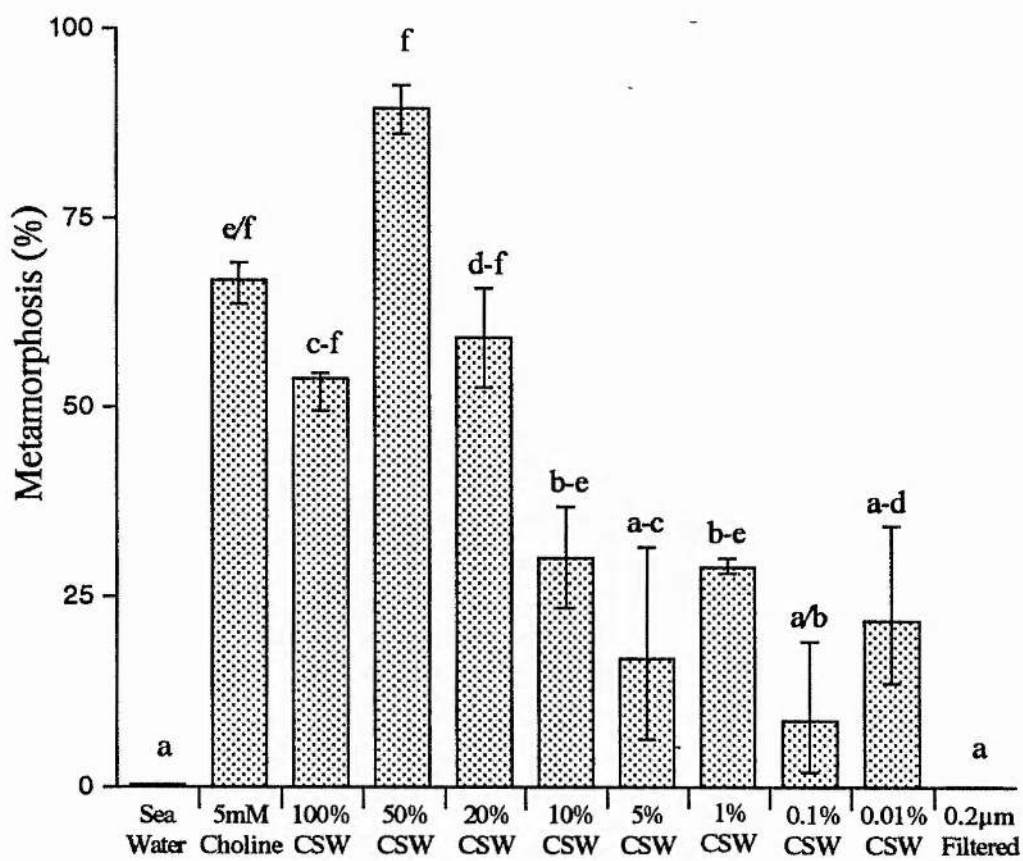
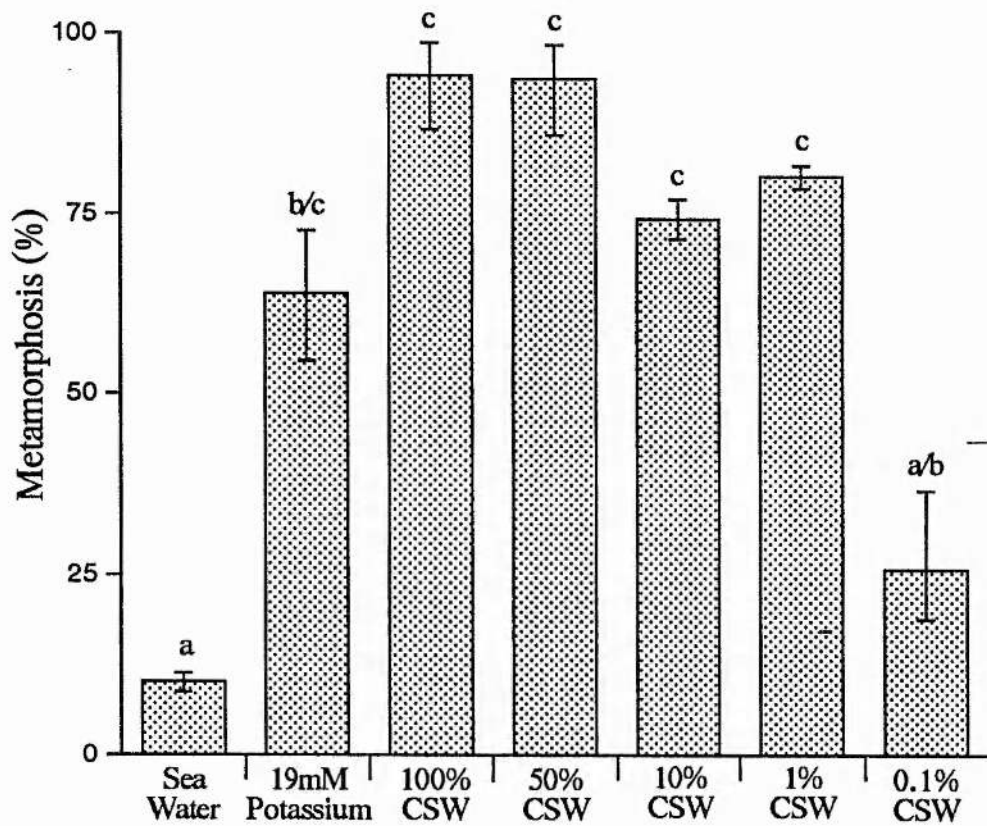
The trends apparent in Experiment 11 are similar to those previously documented for Experiment 8. For example, the maximum level of naturally

**Table 4.5. The effect of serial dilution on the morphogenic capacity of *E. pilosa* CSW.** Relative concentration of CSW (displayed in treatments [D] - [O]) is expressed as a percentage of original strength where 100% = undiluted. Data are back-transformed mean metamorphic responses for each quintuplicate treatment (except Experiments 8 - 10 which were triplicated) expressed as percentages. Multiple comparisons performed within each experiment are illustrated graphically by Figures 4.4 a-e.

Treatment		Experiment				
		8	9	10	11	12
[A]	Sea Water	10.1	0.3	0	4.8	0
[B]	19mM Potassium	63.9	-	-	-	-
[C]	5mM Choline	-	66.7	97.2	90.8	<u>84.4</u>
[D]	CSW 100%	94.2	53.8	40.8	64.5	37.9
[E]	50%	93.6	89.5	34.8	72.2	36.7
[F]	25%	-	-	-	39.8	23.2
[G]	20%	-	59.2	27.8	-	-
[H]	10%	74.2	30.1	16.9	27.0	20.9
[I]	5%	-	16.9	-	-	6.4
[J]	1%	80.2	28.9	6.3	31.9	3.4
[K]	0.5%	-	-	-	-	4.8
[L]	0.1%	25.8	8.7	1.5	19.3	2.5
[M]	0.01%	-	21.9	13.0	25.9	7.3
[N]	0.001%	-	-	2.1	24.6	-
[O]	0.0001%	-	-	-	15.9	-

**Figure 4.3 a. (above right) Experiment 8.** Induction of metamorphosis by a range of *E. pilosa* CSW serial dilutions. Data are back-transformed means and associated standard errors (n=3 replicates per treatment). Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P < 0.05$  significance level.

**Figure 4.3 b. (below right) Experiment 9 .** Induction of metamorphosis by a range of *E. pilosa* CSW serial dilutions. Data are back-transformed means and associated standard errors (n=3 replicates per treatment). Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P < 0.05$  significance level.





induced metamorphosis (72.2%, Table 4.5, row [E]) was triggered by the 50% CSW treatment in a similar manner to Experiment 8. In addition, the lowest CSW concentration to elicit a significant response was the 1% CSW treatment (also apparent in Experiment 8) which produced just 31.9% metamorphosis (Table 4.5, row [J]). Given the demonstrated activity of 1% CSW the non-significant level of metamorphosis triggered by the 10% CSW treatment was unexpected. Multiple comparison by Tukey's HSD did, however, establish that there was no significant difference between the metamorphic responses of all treatments between 25% and 0.0001% CSW, including 10% and 1% CSW (see Figure 4.3 e.).

The maximal naturally induced level of metamorphosis in Experiment 12 was 37.9% (Table 4.5, row [D]) which was elicited by the undiluted CSW treatment. Whilst serial dilutions between 100% and 0.01% CSW were utilized, the most diluted treatment to trigger a significant metamorphic response was 10% CSW (20.9%, Table 4.5, row [H]). Multiple comparisons by Tukey's HSD (the resultant groupings of which are displayed above appropriate columns in Figure 4.3 e.) established there to be no significant difference between the levels of metamorphosis triggered by any of those CSW treatments considered to elicit a significant response (ie 100%, 50%, 25% and 10% CSW treatments). Although the responses of all treatments between 5% and 0.01% inclusive were not considered to be significant ( $P < 0.05$  level), Figure 4.3 e shows them to lie within the same Tukey grouping as the 25% and 10% CSW treatments.

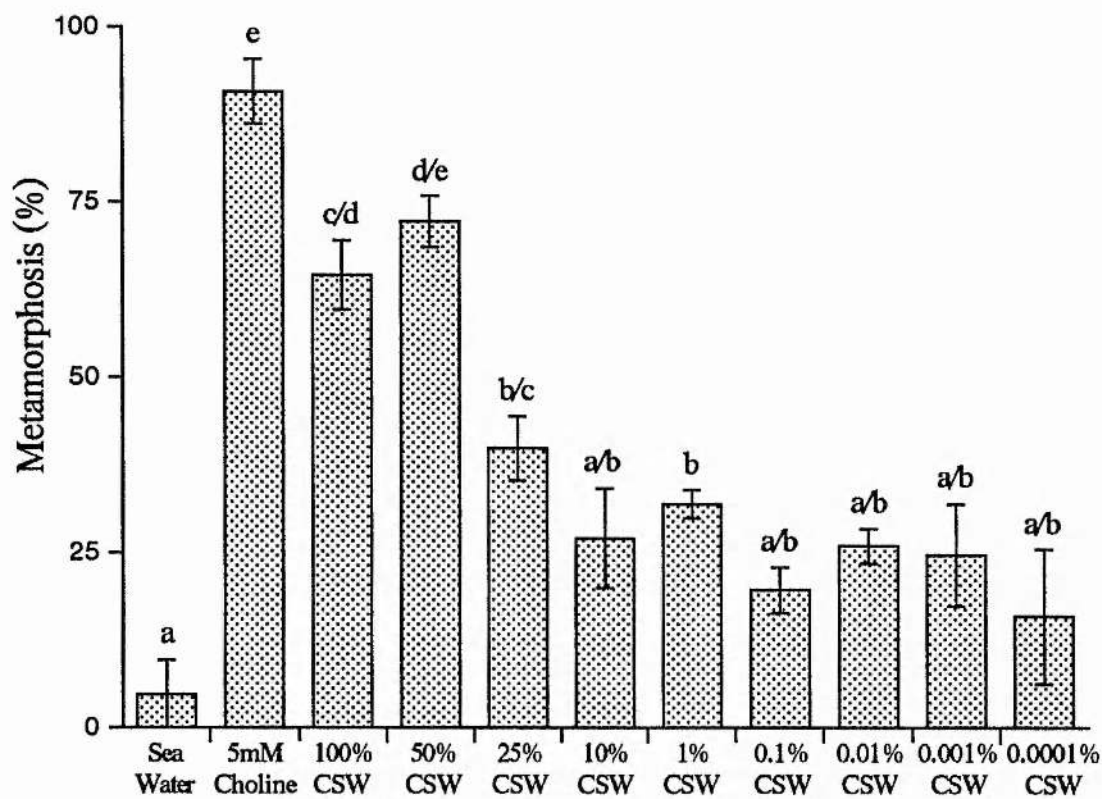
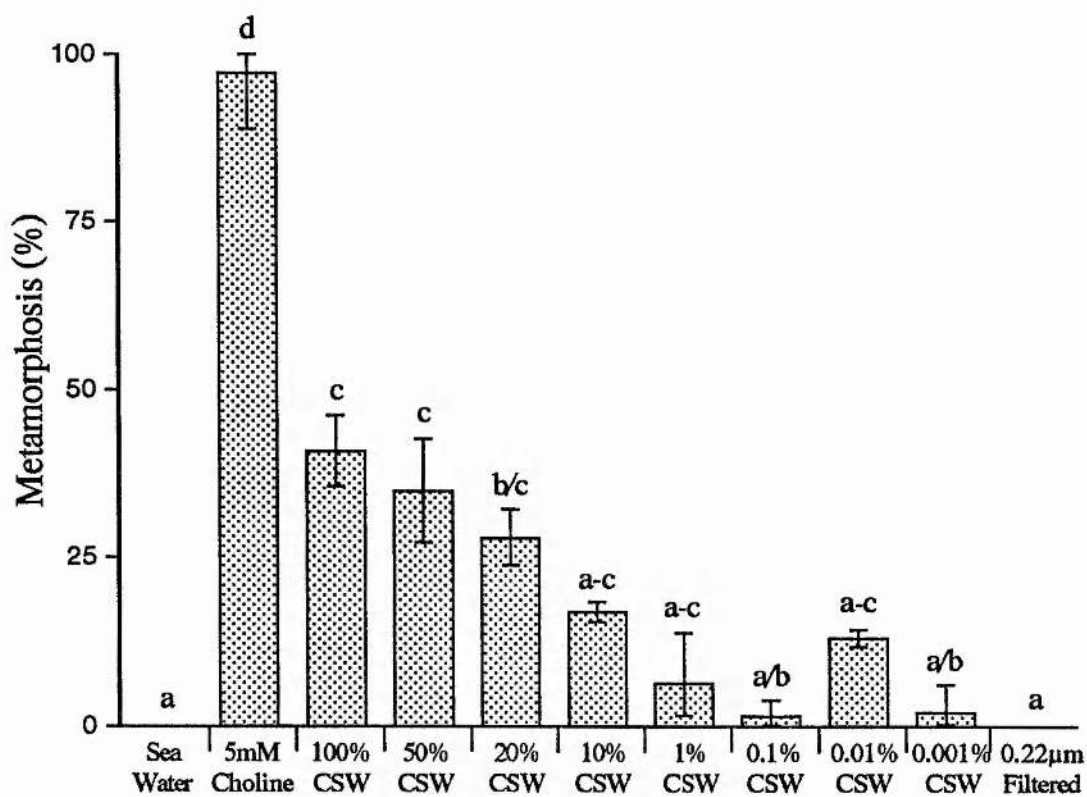
Figure 4.3 f. appears to show a general trend of decreased metamorphosis with increasing dilution, and the maximum level of naturally induced metamorphosis to be consistently attributable to either 100% or 50% CSW treatments. Statistical analysis of individual experiments by one-way ANOVA and subsequent Tukey's HSD established that there was no significant difference evident in any experiment between the levels of metamorphosis elicited by 100 % CSW and 50% CSW treatments. Further, in Experiments 9, 10 & 11 no significant difference was established between any CSW treatments considered to trigger a significantly greater response than the sea water control.

Whilst some CSW treatments triggered metamorphic responses not significantly higher than unconditioned sea water, not one of these considered significantly lower than those CSW treatments which produced a response significantly greater than the sea water control (see Tukey groupings displayed above appropriate columns in Figures 4.3 a-e). For example, the most diluted

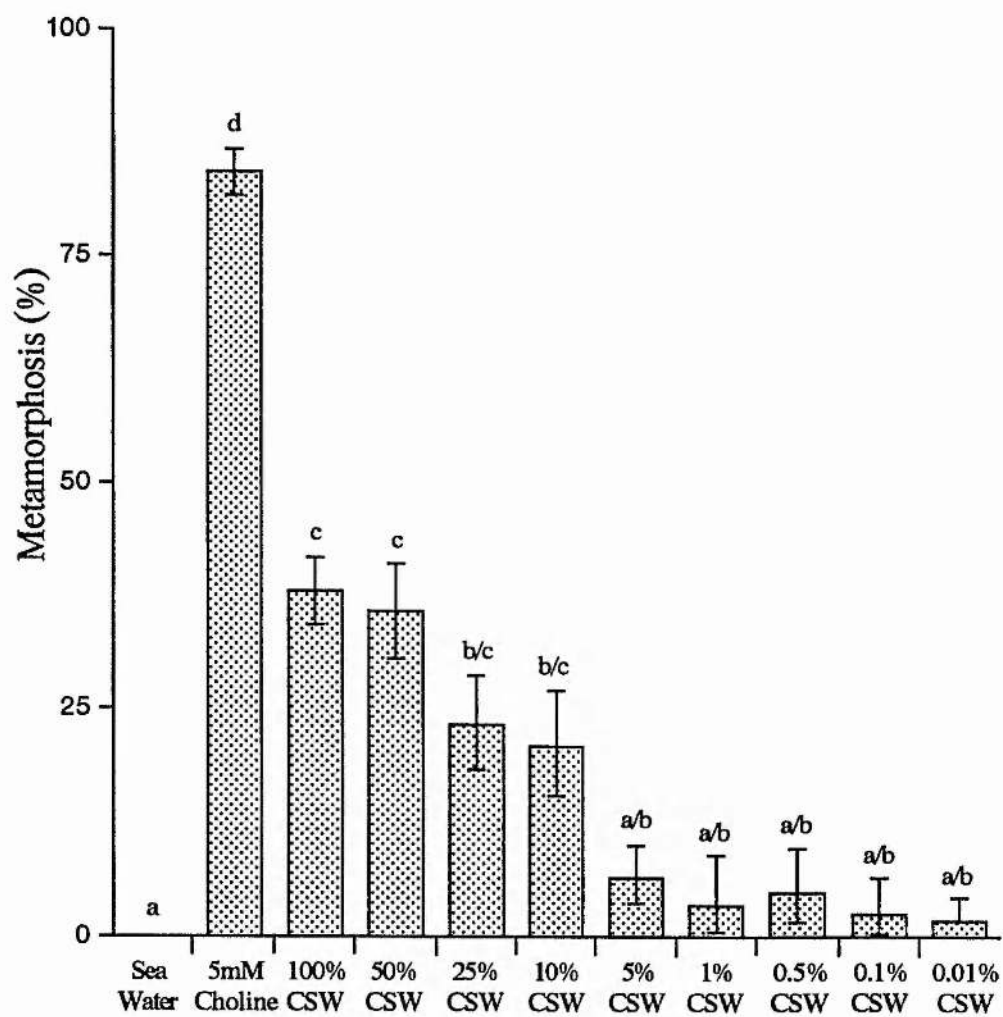
**Figure 4.3 c. (above right) Experiment 10 .** Induction of metamorphosis by a range of *E. pilosa* CSW serial dilutions. Data are back-transformed means and associated standard errors (n=3 replicates per treatment). Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P < 0.05$  significance level.

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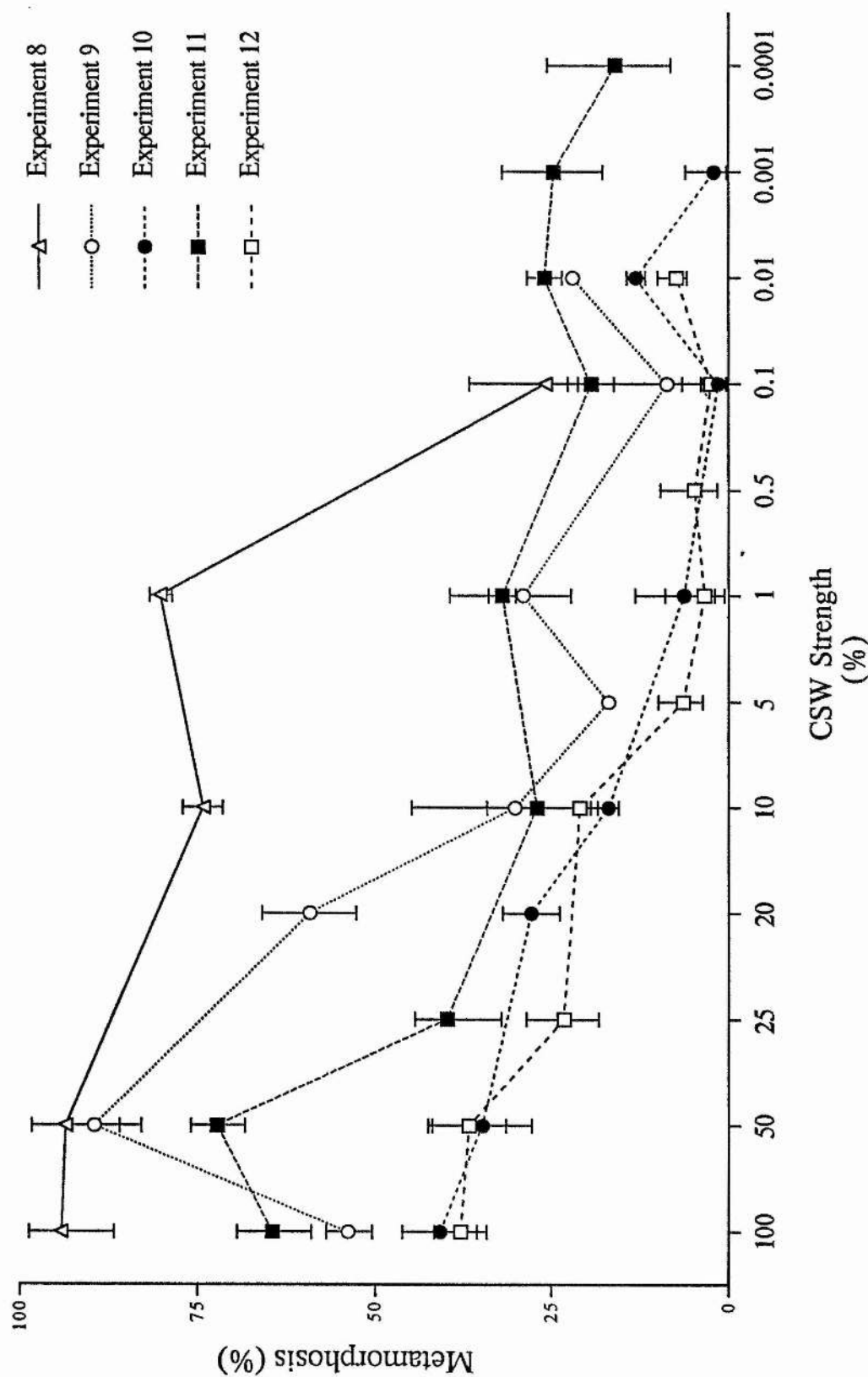
**Figure 4.3 d. (below right) Experiment 11 .** Induction of metamorphosis by a range of *E. pilosa* CSW serial dilutions. Data are back-transformed means and associated standard errors (n=5 replicates per treatment). Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P < 0.05$  significance level.



**Figure 4.3 e. Experiment 12.** Induction of metamorphosis by a range of *E. pilosa* CSW serial dilutions. Data are back-transformed means and associated standard errors (n=5 replicates per treatment). Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P < 0.05$  significance level.



**Figure 4.3 f.** Experiments 8 - 12. The metamorphic responses of larvae on exposure to a range of *E. pilosa* Conditioned Sea Water (CSW) serial dilutions. Points are back-transformed mean metamorphic responses expressed as percentages for each quintuplicate treatment (except Experiments 8-10 which were triplicated) with associated standard error bars. CSW concentrations ranged from undiluted (100% CSW) down to 0.0001% of initial CSW strength. Dilution of CSW was performed using filtered sea water. For the absolute values of back-transformed means and for statistical analysis see Table 4.5. Appropriate Tukey groupings within each experiments are displayed in Figures 4.3 a-d.





treatment tested was 0.0001% in Experiment 11 (see Table 4.5, row [O]). Whilst this treatment triggered a non-significant response of 15.9%, this was in the same Tukey group as the 25% CSW treatment which elicited a significant response of 39.8% (see Figure 4.3 d.). An additional, although extreme, example is provided by the 0.1% CSW in Experiment 10 which triggered the lowest level of metamorphosis of any CSW treatment of just 1.5% (Table 4.5, row [L]). This was considered neither statistically higher than the sea water control, nor lower than significant response of 27.8% triggered by 20% CSW (see Figure 4.3 c.).

#### Other Properties of the Metamorphic Cue

The effects of filtering, treatment with antibiotics, and temperature on the morphogenic capacity of *E. pilosa* CSW are displayed in Table 4.6. Appropriate Tukey groupings within each experiment are indicated by superscript type.

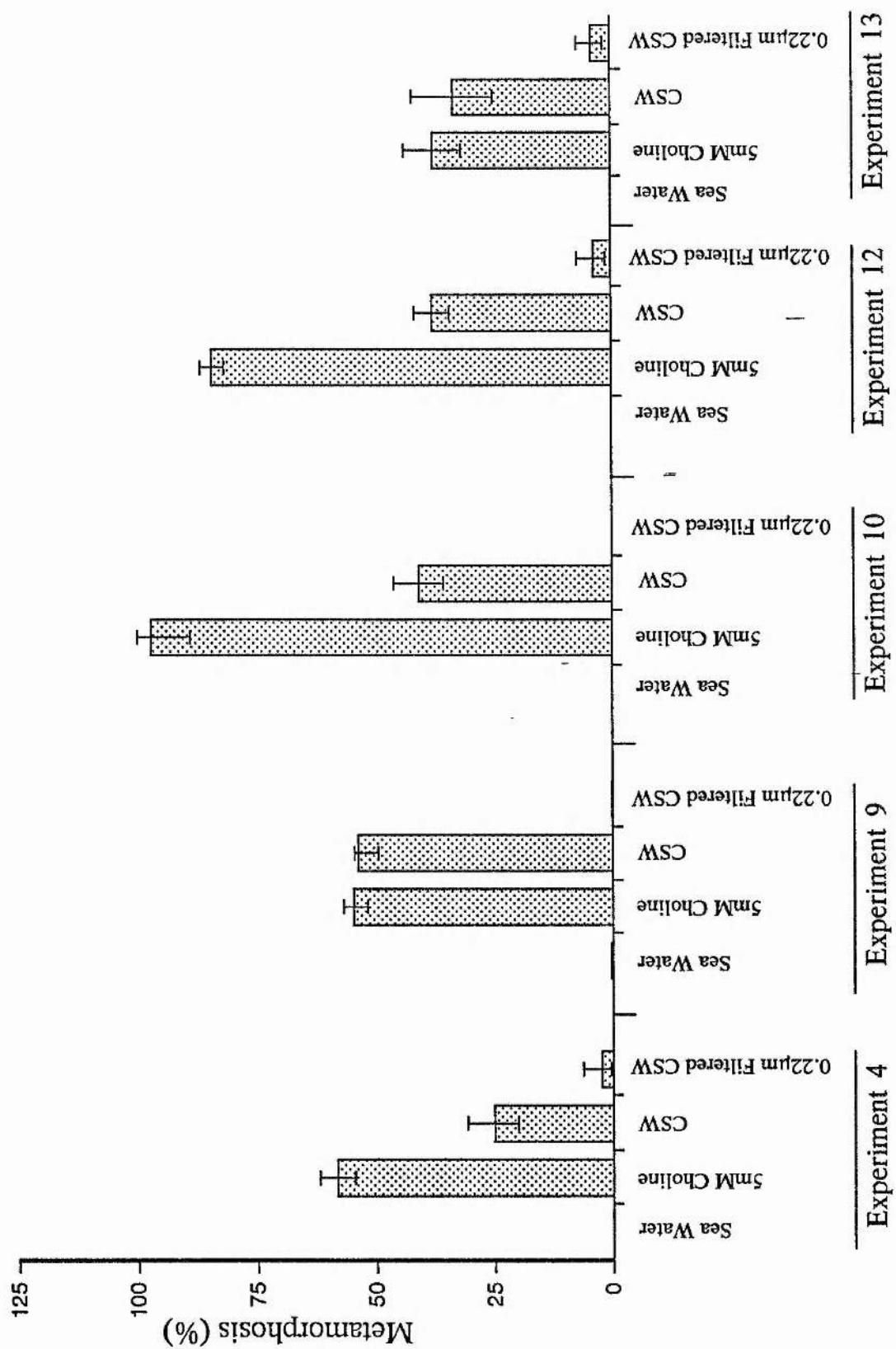
In all Experiments (4, 9, 10, 12 & 13) in which active CSW was 0.22 $\mu$ m filtered, morphogenic activity was not only significantly reduced, but completely eliminated. The metamorphic responses elicited by CSW and corresponding 0.22 $\mu$ m filtered CSW are illustrated by Figure 4.4 a. Veliger competence was confirmed in all five experiments by the significant levels of metamorphosis demonstrated in response to 5 $\cdot$ 10<sup>-3</sup>M choline (Table 4.6, row [B]). The 'active' morphogenic status of the CSW before 0.22 $\mu$ m filtering was confirmed by the inclusion of a CSW treatment in every experiment and significant metamorphic responses were demonstrated throughout (see Table 4.6, row [D]). Whilst metamorphosis in response to CSW varied from 64.5% in Experiment 11 (Table 4.6, row [C]) to 20.2% in Experiment 5 (Table 4.6, row [C]), no such variation was apparent in the 0.22 $\mu$ m filtered treatments. Experiment 13 produced the maximum response of 4.0% (Table 4.6, row [D]) attributable to 0.22 $\mu$ m CSW, whilst the minimum was 0% (Experiment 10, Table 4.6, row [D]).

Filmed surfaces and antibiotic treatments were investigated in Experiments 1, 13 & 14. Experiment 1 investigated the possible influence of bacterial films in the induction of *A. proxima* veliger metamorphosis. Figure 4.4 b. illustrates the lack of metamorphosis observed in larvae on subjection to *E. pilosa* skeletons conditioned both in the presence of *F. serratus* exclusively and in the presence of *F. serratus* and *E. pilosa*. No metamorphosis was observed in response to *E. pilosa*

**Table 4.6.** The effects of filtering, treatment with antibiotics, and temperature on the morphogenic capacity of *E. pilosa* CSW. Data are back-transformed mean metamorphic responses for each quintuplicate treatment (except Experiments 1, 9 & 10 which were triplicated) expressed as percentage responses. Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P < 0.05$  significance level.

[illegible]

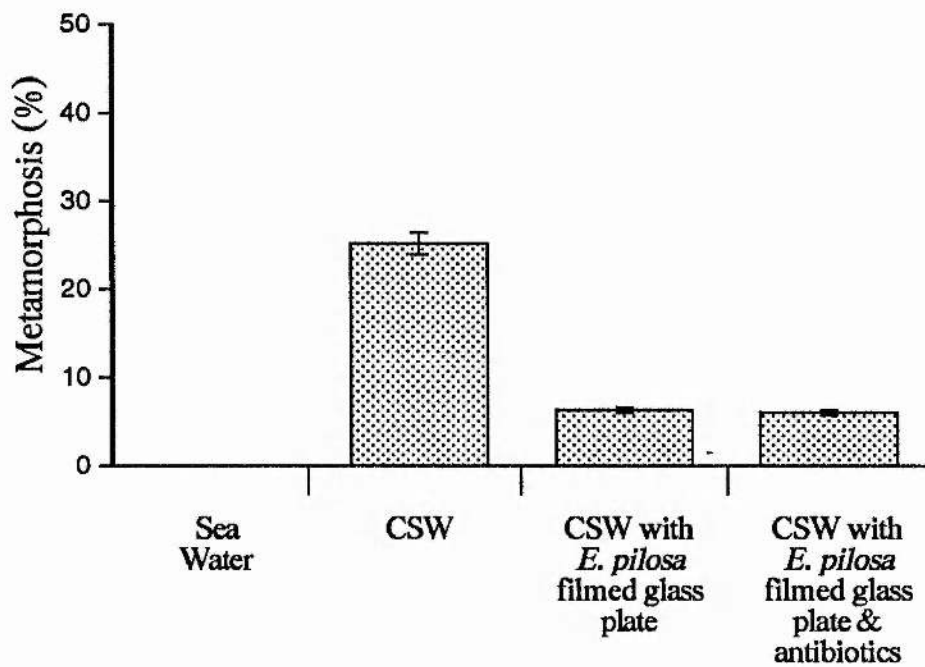
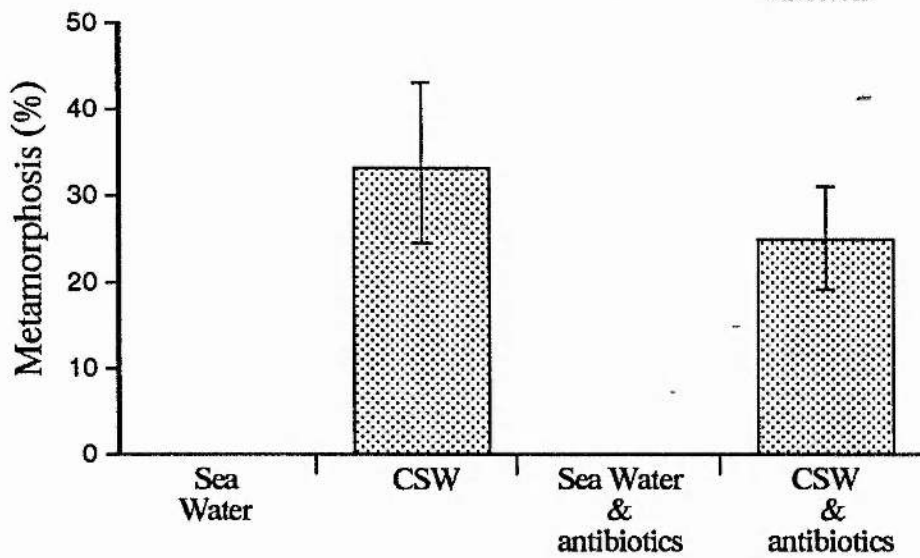
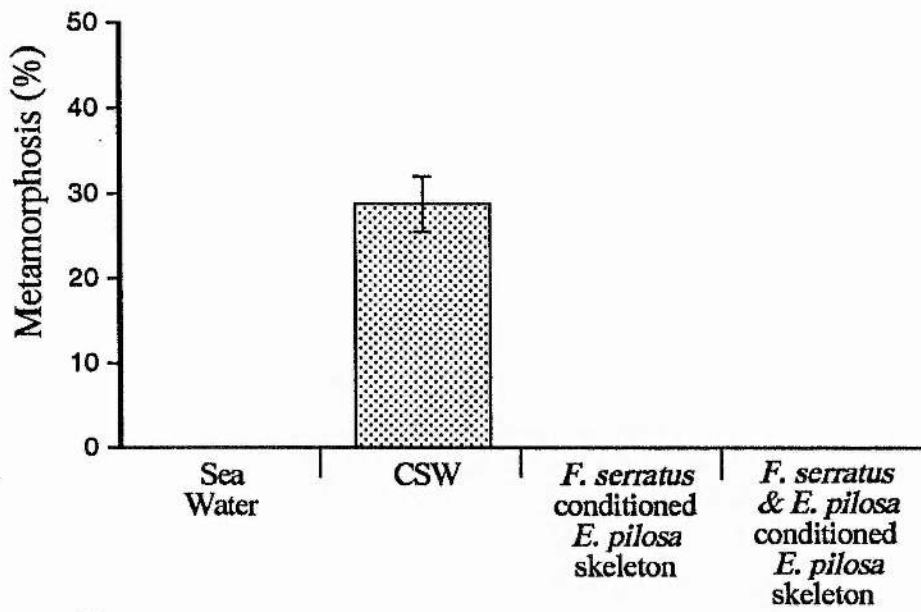
**Figure 4.4 a.** Experiments 4, 9, 10, 12 & 13. The effect of 0.22 $\mu$ m filtering on the morphogenic capacity of *E. pilosa* CSW. Data are back-transformed means and associated standard errors (treatments were quintuplicated except in Experiments 9 & 10 where treatments were triplicated). Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P < 0.05$  significance level.



**Figure 4.4 b. (top right) Experiment 1. The effect of bacterial filming on metamorphosis.** Data are back-transformed means of the triplicated treatments with associated standard error bars displayed. Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P < 0.05$  significance level.

**Figure 4.4 c. (middle right) Experiment 13. The effect of antibiotics on *E. pilosa* CSW.** Data are back-transformed means of the quintuplicated treatments with associated standard error bars displayed. Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P < 0.05$  significance level.

**Figure 4.4 d. (bottom right) Experiment 14. The effect of antibiotics on *E. pilosa* conditioned glass plates.** Data are back-transformed means of the quintuplicate treatments with associated standard error bars displayed. Superscript type denotes groupings resulting from multiple comparisons by Tukey's HSD performed on each experiment. Groupings are considered different at the  $P < 0.05$  significance level.





skeletons conditioned by *F. serratus* (Table 4.6, row [J]). More notable are the disparate results of the *E. pilosa* conditioned *E. pilosa* skeleton treatment which showed no morphogenic capacity (Table 4.6, row [K]) and the CSW resulting from this conditioning treatment which triggered a significant metamorphic response (Table 4.6, row [C]).

The effects of antibiotic treatment on CSW itself were investigated in Experiment 13 (see Figure 4.4c.). The CSW treatment used triggered 33.2% (Table 4.6, row C) of veligers to metamorphose, whilst the sea water + antibiotic control had no significant effect (Table 4.6, row [H]). Whilst the addition of antibiotics to the active CSW produced a lesser degree of metamorphosis than evident in the CSW control (25.0% compared to 33.2%) subsequent multiple comparisons by Tukey's HSD established that this did not constitute a significant decrease in CSW activity (Table 4.6, row [I]).

Further investigation of the morphogenic properties of filmed surfaces and the effects of antibiotics was performed in Experiment 14 (see Figure 4.4 d.). Treatments [L] and [M] consisted of glass petri dishes conditioned in sea water in the presence of live *E. pilosa*. That the sea water was effectively conditioned with the active cue was confirmed by the significant response of 25.2% metamorphosis (Table 4.6, row [C]) observed in response to the CSW treatment. Competent veliger larvae were subjected to these filmed dishes containing active CSW only (Treatment [L]) or active CSW with antibiotics added (Treatment [M]). Whilst the responses (6.4% and 6.1% respectively) of treatments [L] and [M] appeared lower than the 25.2% metamorphosis triggered by the CSW only treatment, Tukey's HSD established there to be no significant difference between the metamorphic responses triggered by any of these treatment responses (see Table 4.6, rows [C], [L] and [M]).

#### Thermal Stability of the Metamorphic Cue Molecule

Heating of CSW to 100°C reduced activity from 37.9% (Experiment 12, Table 4.6., row [C]) to 4.8% (Table 4.6, row [G]). Whilst this constituted a non-significant response at the  $P < 0.05$  level, Tukey's HSD established it to be no lower than the significant response of 23.2% metamorphosis elicited by CSW frozen for 12 h (Table 4.6, row [G]).

Aliquots of fresh CSW from Experiments 5, 10, 11 & 12 were taken and frozen to -20°C for 12h. The morphogenic properties of the fresh CSW were confirmed by the significant levels of metamorphosis exhibited in each of these experiments (see Table 4.6, row [C]). The resulting metamorphic responses of the frozen subsamples are illustrated by Figure 4.4 e. CSW frozen for 12h triggered a significant metamorphic response only in Experiments 11 & 12 (Table 4.6, row [E]). In each experiment there appeared a trend of decreased metamorphosis in response to frozen samples compared to that of fresh CSW controls (see Figure 4.4 e.), although only in Experiment 10 did the frozen CSW sample induce a significantly lower metamorphic response than the CSW control.

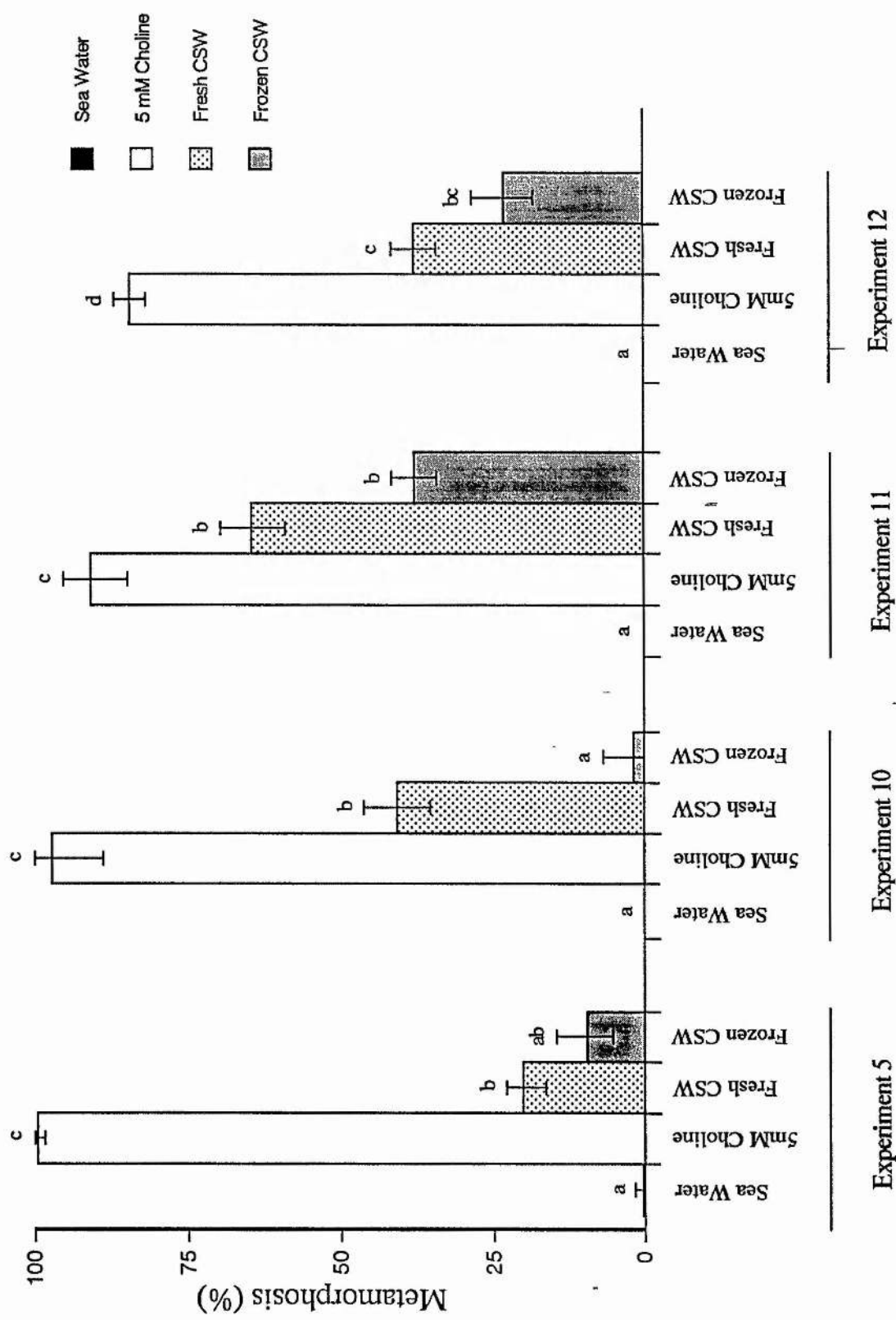
Table 4.6 shows CSW frozen at -20°C for 11d (Experiment 12, row [F]) triggered 50.0% metamorphosis. Although this sample originated from the fresh CSW treatment of Experiment 11 (Table 4.6, row [C]) the prolonged freezing period meant that the 11d frozen CSW treatment could not be run concurrently with Experiment 11 and therefore was included in Experiment 12. Comparison with the morphogenic capacity of the original fresh CSW sample in Experiment 11 was therefore required to evaluate the effect of prolonged freezing on the activity of the metamorphic cue. Multiple pairwise comparison by Tukey's HSD revealed no significant difference between the morphogenic capacities of the corresponding CSW (64.5%, Table 4.6, row [C]), 12h frozen CSW (37.9%, Table 4.6, row [E]) and 11d frozen CSW (50.0%, Table 4.6, row [F]) treatments.

#### Extraction of the Metamorphic Cue Molecule

Experiment 15 constituted an investigation into the morphogenic properties of the aqueous (non-lipophilic) component of CSW. Control treatments of sea water, 19mM potassium and CSW were freshly prepared in addition to the aqueous fractions of *F. serratus* CSW, *E. pilosa* CSW, and *F. serratus* + *E. pilosa* CSW. Minimal mortality was noted for the sea water, *F. serratus* + *E. pilosa* CSW, and 19mM potassium controls (0%, 0% and 1.1% respectively).

In contrast, total mortality (100%) occurred within 24h following initiation of Experiment 15 in all aqueous fraction treatments. *A. proxima* larvae have been previously documented as sensitive to increased ionic potassium concentration in the external medium (Todd *et al.* 1991). It was postulated that the atypically high

**Figure 4.4 c.** Experiments 5, 10, 11 & 12. The effect of freezing on *E. pilosa* CSW. Fresh CSW was frozen (-20°C) overnight, thence defrosted at ambient (10°C) before bioassay. Data are back-transformed means of the quintuplicate treatments with associated standard error bars displayed. Superscript type denotes groupings resulting from multiple comparisons performed by Tukey's HSD on each separate experiment. Groupings are considered different at the  $P < 0.05$  significance level.



mortality may have therefore been attributable to the addition of 1% potassium chloride (0.13 M) used in the washing of the organic layer during lipid extraction. The addition of potassium chloride was therefore excluded from the lipid extraction protocol in subsequent experiments.

The morphogenic properties of both aqueous and organic CSW fractions investigated in Experiment 16 are presented in Table 4.7 with appropriate Tukey groupings and are illustrated by Figure 4.5 a. Larval competence was confirmed by the significant metamorphic response to  $5 \cdot 10^{-3}$ M choline (39.3%, row [B]). The significant level of metamorphosis triggered by CSW (29.5%, row [C]) likewise confirmed the presence of the natural metamorphic inducer in CSW prior to lipid extraction.

No other treatments were found to elicit significant levels of metamorphosis (see Table 4.7, rows [D], [E], [F] & [G]), although the organic CSW fraction did trigger the highest level of metamorphosis (5.7%, Table 4.5, row [G]) observed in all aqueous and organic sea water or CSW treatments. Whilst the metamorphic response of the organic CSW fraction was not considered significant at the  $P < 0.05$  level, multiple pairwise comparison by Tukeys HSD established it to be no lower than that elicited by fresh CSW (Table 4.7, row [C]).

The corresponding mortality data for Experiment 16 (see Table 4.5 and Figure 4.7b.) reveals larval mortality to be statistically negligible in all treatments outwith the lipid extraction procedure (sea water,  $5 \cdot 10^{-3}$ M choline, and CSW). Conversely, both the aqueous fractions of sea water (row D)) and CSW (row [E]) induced significant mortality among larvae (57.7% and 42.0% respectively) as did the sea water organic fraction (42.5%, Table 4.7, row [F]).

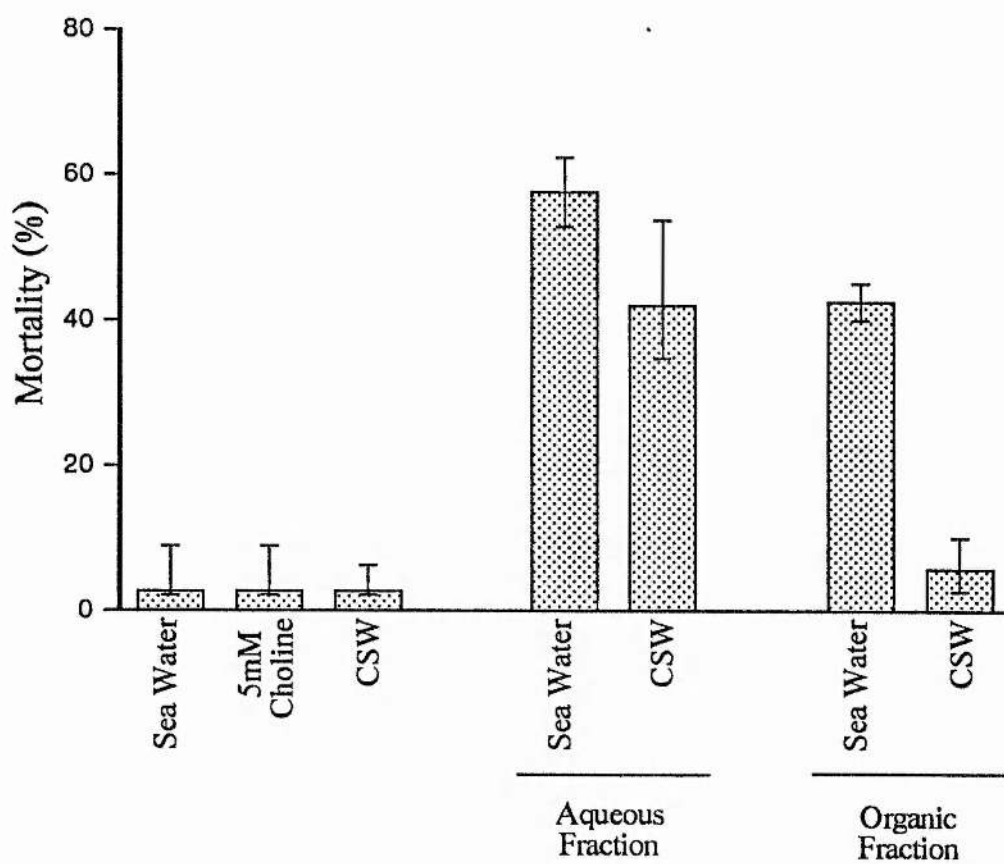
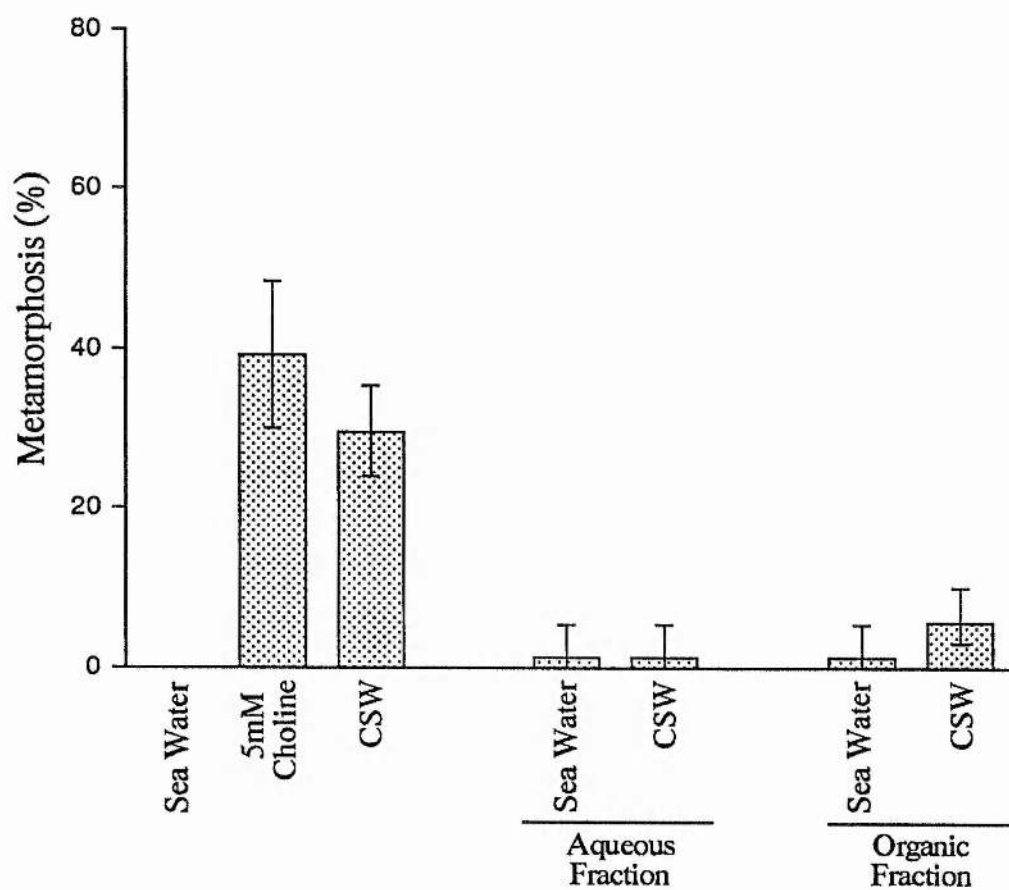
**Table 4.7. Experiment 16. The morphogenic capacity of aqueous and organic fractions of CSW.** Aqueous [treatments D & E] and organic [treatments F & G] fractions were obtained by lipid extraction of CSW using a modified Folch method. Data are back-transformed mean metamorphic responses for each quadruplet treatment expressed as percentage responses. Superscript type denotes groupings resulting from multiple comparisons by separate Tukey's HSD performed on metamorphic and mortality data. Groupings are considered different at the  $P < 0.05$  significance level.

Treatment		Back-Transformed Mean Response (%)	
		Metamorphosis	Mortality
[A] Sea Water		0 <sup>a</sup>	2.6 <sup>A</sup>
[B] 5mM Choline		39.3 <sup>c</sup>	2.6 <sup>A</sup>
[C] CSW		29.5 <sup>b/c</sup>	2.6 <sup>A</sup>
Aqueous Fraction			
[D]	Sea water	1.3 <sup>a</sup>	57.7 <sup>B</sup>
[E]	CSW	1.3 <sup>a</sup>	42.0 <sup>B</sup>
Organic Fraction			
[F]	Sea water	1.3 <sup>a</sup>	42.5 <sup>B</sup>
[G]	CSW	5.7 <sup>a/b</sup>	5.7 <sup>A</sup>



**Figure 4.5 a.** (above) Experiment 16. The morphogenic effect of aqueous & organic CSW fractions on *A. proxima* larvae. Data are back-transformed means of the quadruplet treatments with associated standard error bars displayed.

**Figure 4.5 b.** (below) Experiment 16. The effect of aqueous & organic CSW fractions on larval *A. proxima* mortality. Data are back-transformed means of the quadruplet treatments with associated standard error bars displayed.



## 4.4 DISCUSSION

### Specificity of the Natural Cue

The putative relationship between catholicity of diet and degree of metamorphic cue specificity displayed by marine invertebrates has been examined by both Pawlik (1992) and Havenhand (1991). Sufficient exceptions are apparent (see Table 4.2) to regard any such general link between a stenophagous adult diet and highly specific metamorphic cue requirements with caution. For example *Hypselodoris infucata*, a dorid nudibranch, preys on the sponge genera *Dysidea* sp. (Hubbard, 1988). Whilst the adult exhibits a stenophagous diet, the morphogenic cue requirements of this species exhibit no such specificity. Hubbard (1988) established that veliger larvae will metamorphose in response to a range of non-prey sponge species (see Table 4.2). Neither is this an atypical example. Pawlik (1989) established that larvae of the herbivorous aplysiid *Aplysia californica* metamorphose in response to several species of macroalgae in a non-specific manner. In marked contrast, the adult displays a highly specific diet. Pawlik (1992) rationalized this by proposing that juveniles may locate the adult food source only after metamorphosis. Additional evidence that the link between diet and morphogenic cue specificity must be regarded with caution is provided by the sea star *Mediaster aequalis*. Contrary to all previously cited examples, this species has a catholic adult diet. Rather than exhibiting low specificity in settlement requirements, as might be expected, Birkeland *et al.* (1971) found the larvae to be highly selective, with metamorphosis only on the tubes of the polychaete *Phyllochaetopterus* sp.

Other putative examples of stenophagous species exhibiting specific metamorphic cue requirements may be artefacts of the absence of systematic testing of other possible morphogenic substances in trials (Havenhand, 1991). Thus, whilst the adult prey species may correctly be cited as an effective metamorphic inducer, the specificity of the cue remains undetermined. Such an example is supplied by Carroll & Kempf (1990) in a study of the nudibranch *Berghia verrucicornis*. Whilst it was established that the adult prey anemone species *Aiptasia pallida* does indeed induce larvae to settle and metamorphose, no

alternative possible morphogenic stimuli were presented. This case cannot therefore be presented as indicative of highly specific cue requirements displayed by a stenophagous species. Applying the same rationale, whilst the dorid nudibranch *Onchidoris muricata* has been established by Todd & Havenhand (1985) to metamorphose in response to the adult prey (the bryozoan *Electra pilosa*), the degree of cue specificity displayed by *O. muricata* is unknown.

Conversely, where a range of ecologically relevant morphogenic substances have been tested, the specificity of the cue may be stated with a greater degree of confidence. For example the nudibranch *Phestilla sibogae* has an adult diet composed exclusively of the coral *Porites* sp. Hadfield (1977) found the morphogenic activity of conditioned water and aqueous extracts of other locally common corals (*Pocillopora damicornis* and *Montipora verrucosa*) relative to the prey species *P. compressa* to be largely ineffective. Hadfield (1977) concluded that *P. sibogae* does indeed display a high degree of cue specificity as might be expected of a stenophagous species. That the cue specificity was not absolute was explained by either the production of the cue in low quantities by other corals triggering low levels of metamorphosis or structurally similar compounds mimicking the cue substance emanating from *P. compressa*. In his review of the behaviour of opisthobranch larvae Havenhand (1991) found only one case, that of *Doridella obscura* (Perron & Turner, 1977), in which the putative cue specificity of a stenophagous nudibranch was confirmed by a lack of metamorphic response to other ecologically relevant possible morphogenic sources.

Whilst *Adalaria proxima* exhibits a relatively stenophagous adult diet (Thompson, 1958a; Todd, 1981; Todd *et al.*, 1991; Lambert & Todd, 1994) the specificity of cue requirements in this species have not been previously established. Consequently it was considered a pertinent aim within this study to establish the degree of metamorphic cue specificity displayed by *A. proxima* larvae.

With the notable exception of the cheilostome bryozoan *Electra pilosa*, no other ctenostome or cheilostome bryozoan included in this investigation elicited a significant metamorphic response among competent *A. proxima* larvae. The cheilostome bryozoan *Membranacea membranacea* and ctenostome bryozoans *Flustrellidra hispida*, *Alcyonidium gelatinosum* and *A. hirsutum* all failed to exert any morphogenic effect on competent *Adalaria proxima* veliger larvae either by direct contact or by the conditioning of seawater. This is in marked contrast to the consistently significant levels of metamorphosis evident in response to the

cheilostome *E. pilosa*. Whilst the metamorphic cue specificity of *A. proxima* has not been previously documented, Thompson (1958a) remarked upon the lack of settlement behaviour observed in response to *M. membranacea* and *F. hispida*, although no supporting experimental data were presented. The absence of morphogenic properties demonstrated in the present study by *M. membranacea* and *F. hispida* do not conflict with, and indeed may be considered to provide further support for, the observations of Thompson (1958).

Other possible morphogenic cue sources also proved ineffective in inducing metamorphosis. There are no previous reports within the literature of nudibranch spawn mass jelly possessing morphogenic properties, and *A. proxima* stroma proved no exception. The lack of morphogenic capacity shown by *A. proxima* spawn mass stroma is, however, in contrast with the work of Gibson & Chia (1989a) who established the water-borne substance emanating from the egg mass jelly of another opisthobranch, the cephalaspidean *Haminoea callidegenita*, to induce metamorphosis in both embryos and hatched veligers of that species (previously discussed in Section 3.4).

The ascidian *Botryllus schlosseri* consistently had no effect upon competent *Adalaria proxima* veliger larvae. Levels of metamorphosis obtained on exposure to the sponge *Halichondria panicea* were more inconsistent. Healthy *H. panicea* failed to induce metamorphosis among veliger larvae. On repetition however, 25.2% of larvae unexpectedly metamorphosed (Table 4.3). The sponge used within this experiment had quickly degraded however, causing atypically high mortality (38.0% compared to 4.0% in the sea water control) and discoloured water with a surface film. It would seem likely therefore that the metamorphosis and mortality are attributable to a stress response induced by the irritant by-products of sponge decomposition.

The results of this present study would provide further evidence that, in common with many other stenophagous species, *Adalaria proxima* displays a high degree of cue specificity and may successfully metamorphose in the field only in response to the primary adult prey item, *Electra pilosa*.

### Minimum Effective Exposure Time To The Natural Cue

The minimum exposure time required to effect metamorphosis, when compared to those of other metamorphic cues, may provide information about the nature by which metamorphosis is mediated. Todd *et al.* (1991) performed a comprehensive series of investigations in examining the manner in which artificial metamorphic cues (choline chloride and elevated potassium) induced metamorphosis of *Adalaria proxima*. Competent larvae were exposed to pre-determined optimal doses either of choline ( $10^{-2}\text{M}$ ) or elevated potassium (19mM in artificial seawater) for set time periods of 1 h, 2 h, 7.5 h and 24 h in addition to a continuously exposed treatment. Both artificial cues induced significant levels of metamorphosis, although continuous exposure of competent larvae was required for metamorphosis to be induced by elevated potassium, in contrast to a minimum of between 1-2 h exposure to choline. The authors concluded this disparity in the effective minimum exposure periods to be indicative of the different metamorphic pathways employed by choline and elevated potassium, or that they involved different parts of the same pathway.

The present study investigated the minimum effective response time required for the natural cue to effect metamorphosis in *Adalaria proxima*. No significant difference was found in metamorphic response between the 30 min, 1 h and continuously exposed treatments. The minimum effective exposure time established for the natural cue to successfully initiate metamorphosis was 2 min (Experiment 6). Clearly, when compared with the experimental findings of Todd *et al.* (1991), these results show a disparity in minimum required exposure periods, and by intimation, the manner by which metamorphosis is induced, between elevated potassium and the natural cue. Whilst continual exposure is required for elevated potassium to induce metamorphosis, there is no difference in effect between 30 min and continual exposure to the natural cue. The distinct mechanisms by which elevated concentrations of inorganic ions (potassium), putative neurotransmitters (choline) and natural cues are proposed to mediate metamorphosis have been previously discussed (see Section 3.4.). Whilst the identity of the natural metamorphic inducer remains unknown, its mechanism of action might therefore be reasonably expected to be distinct from that of elevated potassium, and this is indeed reflected by the different effective exposure periods observed.

No significant difference in metamorphic response was established between 2 h, 7.5 h 24 h and continuous exposure periods using choline (Todd *et al.*, 1991)



and the authors concluded that between 1 - 2 h exposure to choline was sufficient to elicit metamorphosis. Given the difference in set exposure times and possible variation in larval response between the present study and that of Todd *et al.* (1991) the minimum effective exposure times of choline and the natural cue may be considered comparable. Whilst a difference in minimum effective exposure time between cues is informative, similar effective exposure periods obviously cannot be interpreted to intimate necessarily similar mechanisms of metamorphic induction.

Comparison of larval responses between cue exposure experiments within the present study reveals considerable variation in metamorphic response to identical exposure periods. Whilst such between-batch variation in larval response renders direct comparisons between experiments inappropriate, a trend of decreased metamorphic response with decreased exposure time is apparent (see Table 4.4). The significance of this decrease in Experiment 7 (see Figure 4.2 b) would intimate metamorphic response to be a function of exposure time.

Biological variation is implicit in the nature of these investigations and similar, albeit less extreme, variation in larval metamorphic response to identical conditions has been consistently observed throughout this study. Considerable variation in response to morphogenic cues has been previously documented in many molluscan larvae (Hadfield, 1977, 1984; Hadfield & Scheuer, 1985; Bonar *et al.*, 1990; Hadfield & Pennington, 1990; Gibson, 1993) and indeed, in the species under study (Todd *et al.*, 1991). Such variation has been attributed to between batch differences in larval sensitivity, or threshold requirement, to the metamorphic cue (Hadfield, 1977, 1984; Bonar *et al.*, 1990). Comparison of metamorphic response between experiments where this source of variation is considerable cannot be made with any high degree of confidence (Bonar *et al.*, 1990).

### Potency of the Metamorphic Cue

The natural metamorphic cue of *Adalaria proxima* has been established to be a water-borne substance emanating from the primary adult prey *Electra pilosa* (Thompson, 1958a; Lambert & Todd, 1994). Knowledge of the potency of the natural cue molecule was considered important for several reasons. Further purification of the unknown cue will require repeated bioassays, the reliability of which will be facilitated by an appreciation of the highest effective dilution factor. This is of particular importance for the resuspension of extracted dried fractions



prior to bioassay (for example see Johnson *et al.*, 1991). Relative cue potency may also indicate the effective range of the inducer from a point source, thereby providing some indication of the ecological relevance of the cue. Throughout this series of experiments the maximum metamorphic response was consistently attributable to either undiluted (100%) or 50% CSW treatments. Furthermore, no significant difference between the metamorphic responses elicited by 50% and 100% CSW was established within any of the 5 dilution series.

There is little experimental evidence to suggest a cut-off point or maximum dilution beyond which zero metamorphosis would occur; rather there was a trend of declining metamorphic response with increasing CSW dilution. This is evident from the lack of significant difference evident between the metamorphic responses elicited by successive increasing dilution treatments. This is demonstrated by the Tukey groupings displayed for each experiment above the appropriate columns in Figures 4.3 a - e.

Despite the necessarily qualitative nature of the data, comparisons with the literature reveal markedly similar trends. The present findings concur with the observations of Hadfield (1977) and Hadfield & Pennington (1990) in their investigation into the nature of the larval response of *Phestilla sibogae* to the metamorphic cue, a water-soluble substance emanating from the coral *Porites compressa*. A dilution series performed by Hadfield & Pennington (1990) on the aqueous coral extract revealed the maximum effective concentration to be triggered by undiluted (100%) and 50% diluted extract, and thereafter to decline with subsequent increasing dilutions. Again in a similar trend to that observed in the natural metamorphic cue of *A. proxima*, there was no difference between the metamorphic responses elicited by 50% and 100% coral extract. Neither is this effect confined to prey-specific cues. For example, *Crassostrea gigas* may be triggered to metamorphose in response to bacterial supernatants (Fitt *et al.*, 1990). Separate serial dilutions of supernatants from the bacterial species *Alteromonas colwelliana* and non-pigmented *Vibrio cholerae* established that the maximum metamorphic responses were in response to 50% and 100% initial strength solutions, and that there was no significant difference in the level of response elicited by the 50% and 100% solutions.

Between-experiment variation observed in larval metamorphic response is reflected both in the absolute levels of metamorphosis triggered by identical CSW treatments and in the minimum CSW concentrations required to elicit a significant

level of metamorphosis (see Table 4.5 and Figure 4.3f). The possibility that variation in the conditioning of water resulted in between-experiment differences in initial cue concentration was investigated by scaling the metamorphic responses elicited by each dilution factor as a proportion of the maximum metamorphic response within each experiment. No between-experiment trends in proportional responses to treatments of the same dilution factors were observed. This would imply that the between-experiment variation in metamorphic response observed may not be attributed to differences in initial cue concentration.

The minimum effective CSW concentration required to trigger a significant metamorphic response in competent *Adalaria proxima* larvae was established to be 1% in Experiments 8, 9 and 11, yet was 20% and 10% in Experiments 10 & 12 respectively. It is unlikely that these higher minimum effective concentrations could be attributable to the conditioning protocol itself because this was kept constant throughout. Different batches of *E. pilosa* were, by necessity, used to condition water, and this may have resulted in unquantifiable differences in the concentration of cue present between experiments, although the comparison of proportional metamorphic responses discussed above would not support this. Between batch variation in larval sensitivity to the cue and threshold requirements previously discussed may be a considerable factor in the observed variation in minimum effective CSW concentration.

Such variation in response has been interpreted to indicate the different threshold requirements and sensitivities possessed by larvae (Hadfield, 1977; Hadfield & Pennington, 1990). Whilst larval differences in threshold requirements and sensitivity to the cue may complicate experimental analysis, they have been postulated to be of adaptive significance. Hadfield (1977) proposed such variation in both effective exposure time and cue concentration required to initiate metamorphosis to constitute an adaptive mechanism to ensure the adequate dispersal of progeny. Considerable variation in larval response to identical conditions has been observed both by Todd *et al.* (1991) in their comprehensive investigation of metamorphosis in *Adalaria proxima* and Lambert & Todd (1994) in their investigation into the nature of the metamorphic cue. Whilst Todd *et al.* (1991) acknowledged the possible significance of such variation on distribution, on examination of *A. proxima* population structure, as revealed by genetic data compiled in Todd *et al.* (1988), they found no support that the observed variability in larval response resulted in widespread distribution of progeny within this species.

Reports in the literature quantifying the response of nudibranch larvae to different dilutions of inducer are sparse. In a study of the induction of settlement and metamorphosis in the nudibranch *Onchidoris bilamellata* (see Table 4.2), Chia & Koss (1988) examined the potency of the unknown water-borne settlement inducing agent emanating from the barnacle *Chthamalus dalli*. Although definitive interpretation of the significance of the larval responses appears compromised by restricted sample sizes and statistical information, it is evident that settlement declined in response to conditioned water diluted to less than 2% of former strength and ceased completely below 0.1% of original strength. Whereas this concerns the settlement cue of *O. bilamellata* in contrast to the morphogenic cue of *A. proxima* addressed in this present study, it perhaps noteworthy that the effective potencies of both unknown water-borne nudibranch cues lie within the same order of magnitude.

The absolute potency of metamorphic cues may constitute only imprecise estimates until the identity (and therefore molecular weight) of the cue molecule is identified. Perhaps more is known about the nature of the water-soluble metamorphic cue of *Phestilla sibogae* than any other nudibranch to date. Hadfield & Pennington (1990) estimated the minimum effective concentration of the metamorphic cue of *P. sibogae* to lie between  $5 \cdot 10^{-9} \text{M}$  -  $5 \cdot 10^{-10} \text{M}$ . Whilst derived from both comprehensive investigation into the nature of the cue and on logical assumption, the errors inherent in the process are considerable. Consequently, the accuracy and ecological significance of stating such absolute values may certainly be considered questionable.

#### Bacterial Association of the Natural Cue

Active CSW subjected to  $0.22 \mu\text{m}$  filtering showed not only a significant reduction in, but almost complete elimination of, morphogenic activity. Metamorphic responses were reduced from between 20.2% and 64.5% prior to filtration down to just 0% to 4.0% after filtration. It is not possible to state in absolute terms that the presence of the cue molecule itself was completely eliminated from the filtrate. Rather, it is possible to state that filtering sufficiently decreases the concentration of the cue molecule such that it lies below the effective threshold concentration required by 96 - 100% of *Adalaria proxima* larvae, effectively eliminating the morphogenic capacity of active CSW. The elimination of

morphogenic activity by 0.22 $\mu$ m filtration would intimate the cue molecule to be associated with a relatively large entity present within CSW such as bacteria or organic aggregates.

Bacterial exudates as well as bacteria themselves have been demonstrated to provide the requisite morphogenic stimulus for many marine invertebrates (Weiner *et al.*, 1985; Hofmann & Brand, 1987; Fitt *et al.*, 1990), although this experimental evidence does not support any such conclusion for *Adalaria proxima*. Whilst a 0.22 $\mu$ m filter will retain bacteria, any exudates will pass through, thereby conserving morphogenic activity in the filtrate. Such an example is supplied by McGee & Targett (1989) in their investigation into the water-borne metamorphic cue of the gastropod *Crepidula plana*. Although 0.2 $\mu$ m filtration of adult-conditioned seawater eliminated bacteria, no reduction in morphogenic activity was apparent. The authors therefore proposed the morphogenic molecule to be a metabolite produced by either adult *C. plana* or by bacteria associated with adult *C. plana*. Similarly, Neumann (1979) found suspension cultures of the marine bacteria *Vibrio* sp. were active in triggering larval metamorphosis of the scyphozoan *Cassiopea andromeda*. Millipore filtering (0.22 $\mu$ m) of the active suspension did not reduce the metamorphic response and treatment with an antibiotic mix (penicillin and streptomycin sulphate) was found to inhibit, although not eliminate, metamorphosis. These results were interpreted as showing the morphogenic agent to be an exudate released into the surrounding medium by healthy, actively growing bacteria. Ultrafiltration revealed the bacterial products to be between 1000 and 10,000 Da.

The reduction in metamorphosis observed in response to 0.22 $\mu$ m filtering of CSW in the present study would therefore imply that exudates emanating from bacteria play no part in the metamorphic induction of *Adalaria proxima*. The results of filtration did not define the possible importance of bacteria themselves in metamorphic induction however. Further trials designed to investigate any association between the bacterial fauna associated with *Electra pilosa* and the metamorphic cue provided no evidence of a bacterial role in the metamorphic induction of *A. proxima*. The failure of unconditioned dead *E. pilosa* skeletons to induce metamorphosis has previously been documented (H. White, unpublished, cited in Todd *et al.* 1991). Neither bacterial *E. pilosa* skeletons 'conditioned' in the presence of *F. serratus*, or in the presence of *E. pilosa* and active CSW induced metamorphosis in this present study. The failure of *E. pilosa* skeletons in triggering a metamorphic response contrasted with the significant level of



metamorphosis elicited by the CSW resulting from the conditioning process (Table 4.6). This would further support the hypothesis that the morphogenic cue is present within the water column rather than associated with any bacterial film.

Administration of antibiotics (penicillin & streptomycin sulphate) had no significant effect on the morphogenic capacity of active CSW, providing further evidence that bacteria, or associated exudates, play no role in metamorphic induction.

Similar findings are documented by Pearce & Scheibling (1990a) in their investigation into the water-borne metamorphic cue of the sand dollar *Echinarachnius parma*. Sand conditioned by the prior presence of adults and subsequently treated with the an antibiotic mix of penicillin & streptomycin sulphate showed no difference in morphogenic capacity compared to untreated adult-conditioned substrate. A significant reduction in bacterial numbers confirmed the efficacy of the antibiotic mix administered. This is in contrast to the findings of Johnson *et al.* (1991) who established that treatment of the coralline alga *Lithothamnium pseudosorum* with an antibiotic mix (streptomycin sulphate, penicillin G, and tetracycline) significantly reduced the metamorphic response of *A. planci* larvae. The morphogenic agent of *A. planci* was therefore concluded to be associated with epiphytic bacteria.

#### Thermal Stability of the Metamorphic Cue

Further investigations concerned the heat stability of the metamorphic cue. Marine invertebrates from several taxa have been found to respond to heat sensitive morphogenic cues. For example, Pearce & Scheibling (1990a) established the water-borne metamorphic cue of the sand dollar *Echinarachnius parma* to be a relatively small molecule (<1000 Da) destroyed by heating. A heat labile metamorphic inducing factor has also been implicated for the green sea urchin, *Strongylocentrotus droebachiensis* (Pearce & Scheibling, 1990b). The settlement cue of the nudibranch *Onchidoris bilamellata* (Chia & Koss, 1988) and morphogenic cues of the asteroid *Acanthaster planci* (Johnson *et al.*, 1991) and the polychaete *Phragmatopoma californica* (Jensen & Morse, 1984) have all been demonstrated to be inactivated by heating.

In contrast, several heat stable metamorphic cues have been documented within the Opisthobranchia. For example, the cephalaspidean *Haminoea callidegenita* metamorphoses in response to a water-borne polar cue molecule associated with the egg mass stroma (Gibson & Chia, 1994) which has been established to be thermally stable between -60°C and 100°C as well as acid stable with a size of <1000 Da. The metamorphic cue of *Phestilla sibogae*, also a small (<500 Da) and pH stable (1-10) molecule has also been demonstrated to retain morphogenic capacity after heating to 100°C (Hadfield, 1984).

The metamorphic cue of *Adalaria proxima* appears stable down to -20°C. Freezing of CSW to -20°C for 12 h resulted in a decrease of morphogenic activity compared to that of fresh CSW. Whilst only one of four CSW samples which had been frozen elicited a significant metamorphic response, it is not the absolute metamorphic response which is pertinent in evaluating the effect of freezing on the cue molecule, but rather it is the morphogenic capacity of the frozen CSW compared to that of the appropriate fresh CSW treatments. Statistical comparison established active CSW frozen to -20°C to trigger levels of metamorphosis no lower than those elicited by the corresponding fresh active CSW in three out of four experiments. Neither was freezing of CSW for a prolonged period (11 d) found to significantly decrease the metamorphic capacity of the cue. The results of this study indicate that freezing (to -20°C) has no significant effect on cue morphogenic capacity, although activity may be marginally decreased.

#### Extraction of the Metamorphic Cue

Characterisation of metamorphic cues requires the extraction of the cue molecule from the surrounding medium. Several studies have successfully extracted water-soluble metamorphic cues for subsequent (partial) purification (e.g. Hadfield & Pennington, 1990; Johnson *et al.*, 1991 b; and Gibson & Chia, 1994). Preliminary trials attempting extraction of the water-soluble metamorphic cue of *Adalaria proxima* proved unsuccessful. Assayed fractions, whilst causing atypically high levels of mortality, failed to induce significant levels of metamorphosis. The failure of the extraction may be attributable to several factors. The metamorphic cue may have been present in sub-threshold concentrations, detrimentally affected by some aspect of the extraction procedure or insoluble in the chloroform : methanol organic solvent mix used. For example, Gibson & Chia

(1994) found the morphogenic cue of *Haminoea callidegenita* could be successfully extracted from egg mass stroma only using methanol.

Unsuccessful extraction may be attributable not just to the insolubility of the cue molecule in chosen solvents. For example, in a study of the morphogenic agent of *Acanthaster planci*, Johnson *et al.* (1991) performed a range of solvent extractions of the coralline alga *Lithothamnium pseudosorum*. Freeze-dried and resuspended extracts coated upon coral heads failed to elicit a settlement or metamorphic response. Whilst this may have been attributable to the insolubility of the morphogenic cue those solvents used (water, ethanol and chloroform), the authors concluded that the loss of morphogenic activity apparent in the extracts may alternatively have been caused by the extraction process detrimentally affecting the cue, or that the unknown concentration of extract applied to the coral heads may have been super- or suboptimal.

Evaporation of the solvent during the extraction procedure will result in desiccation of the cue substance. Successful documented partial purifications prove several water-soluble cues to be unaffected by drying (Hadfield & Pennington, 1990; Gibson & Chia, 1994), but it may be that drying of certain cue molecules results in either reduced morphogenic activity or that the cue cannot be successfully redissolved.

No apparent cause of the atypical mortality observed on assay of extracts can be found. It is possible that such mortality may be attributable to incomplete evaporation of solvent, such that larvae were exposed to low concentrations of methanol. Pennington & Hadfield (1989) established that organic solvents encompassing a span of polarities and functional groups had the capacity to induce metamorphosis in competent veliger larvae of the nudibranch *Phestilla sibogae*. Furthermore, optimum concentrations were consistently near the toxic limit for the larvae. Sensitivity to organic solvents was not however, universal; three of 14 organic solvents assayed - ethylene glycol, dimethyl sulfoxide and hexane - failed to elicit any metamorphic response. Similarly, both ethanol and methanol proved ineffective when administered to planulae larvae of the hydroid *Hydractinia echinata* (Berking, 1988).



## Conclusion

The isolation and identification of natural settlement and metamorphic inducers has been previously identified as a research priority (Hadfield, 1986; Chia, 1989; Pawlik, 1990, 1992; Rodriguez *et al.*, 1993). Whilst the sources and effects of natural metamorphic cues on invertebrate larvae have been extensively cited within the literature, further progress has been hampered by a widespread inability to purify and isolate these largely unknown compounds.

This present investigation was designed to compliment previous research on *Adalaria proxima* by further characterizing the nature of the metamorphic cue, and to facilitate the eventual purification and identification of the cue molecule. The results of this study suggest larvae of *A. proxima* metamorphose in a highly cue-specific manner to a morphogenic substance emanating from the primary adult prey species, the bryozoan *E. pilosa*. Natural cue mediated metamorphosis may be effected in a manner distinct from elevated potassium, requiring 30 min exposure in contrast to continual exposure for maximum metamorphic response to occur. Metamorphosis appears dose dependent, being maximum in response to undiluted and 50% diluted *E. pilosa* conditioned sea water, and thereafter declining to a minimum effective concentration of 1% original strength. Whilst 0.22 $\mu$ m filtration significantly reduces morphogenic activity no support was found for a bacterial role in the induction of metamorphosis. This may suggest the cue molecule to be present within the water column in association with larger organic complexes.

## CHAPTER 5

### DELAYED METAMORPHOSIS

#### 5.1 INTRODUCTION

The pelagic or pre-juvenile period between hatching and metamorphosis, during which time a larva is present in the plankton, varies both inter- and intra-specifically among benthic marine invertebrates (Hadfield & Miller, 1987; Pechenik 1990). This period is composed of an obligatory precompetent phase and a facultative subsequent competent period (Pawlik 1992). The duration of the precompetent period is species-specific and will tend to constitute a shorter time in those species which employ lecithotrophic larvae rather than planktotrophic (Chia, 1978b; Pawlik 1992); this has been previously addressed in detail within Section 3.1. Metamorphosis of marine invertebrates is not an endogenously timed occurrence and therefore on attainment of physiological competence a suitable settlement and metamorphic stimulus must be encountered for the shift from planktonic veliger to benthic juvenile to take place (Pawlik 1992). In the absence of such a suitable cue, many species possess the capacity to delay metamorphosis, that is, to remain in the water column whilst retaining the ability to successfully settle and metamorphose (Thorson, 1950; Crisp, 1974; Coon *et al.*, 1990; Pechenik, 1990; Pawlik, 1992). It is this extended competent phase, during which metamorphosis is delayed, which will be addressed within this Chapter. Many groups of marine invertebrates have been documented to possess the capacity to delay metamorphosis, including cnidarians (Fitt *et al.*, 1987; Morse *et al.* 1988), annelids (Butman *et al.*, 1988; Pawlik, 1988; Pechenik & Cerulli, 1989), echinoderms (Strathmann, 1978; Highsmith & Emlet, 1986; Rumrill, 1989), arthropods (Knight-Jones 1953) and urochordates (Olson 1983), bryozoans (Nielson, 1981; Woollacott *et al.*, 1989) and molluscs (Bayne, 1965; Hadfield, 1977; Perron & Turner, 1977; Pechenik, 1984; Pechenik & Lima, 1984; Kempf, 1981; Miller, 1988; Paige, 1988; Pechenik & Eyster, 1989; Coon *et al.*, 1990; Miller, 1993).

### The Ecological Significance of Delay Capacity

An extended pelagic period has the potential to affect such fundamental parameters as the geographic distribution and population structure of a species (Strathmann, 1974; Miller, 1993). The capacity of a species to delay metamorphosis is consequently considered to be of particular ecological significance (Crisp, 1974; Scheltema, 1974; Strathmann, 1974; Pechenik, 1980, 1984, 1985; Crisp, 1986; Coon *et al.*, 1990; Pechenik, 1990; Miller 1993). The ability to withstand a delay in metamorphosis has been postulated to confer increased probability of survival and reproduction by raising the likelihood of encountering favourable habitats associated with increased fitness (Scheltema, 1961; Thorson, 1950; Meadows & Cambell, 1972; Crisp, 1974, 1986; Obrebski, 1979; Coon *et al.*, 1990). The increased potential for larval dispersal resulting from an extended competent period also may confer benefits by allowing the opportunity to colonize temporally unstable or distant habitats (Scheltema 1975), resulting in increased genetic exchange between geographically separate populations (Strathmann & Branscombe, 1979; Scheltema, 1975; Pechenik, 1988). However, an extended pelagic period is not universally advantageous; whilst increased larval dispersal may reduce the probability of local population extinction (Strathmann & Branscombe, 1979) it may also compromise the capacity of a species to adapt to changing local conditions (Palumbi & Wilson, 1990). The increased planktonic period commensurate with increased dispersal potential may also incur varied costs to the individual. These include an increased probability of death by predation (Crisp, 1974; Day & McEdward, 1984) and fluctuating food availability resulting in periods of starvation (Bayne, 1965; Allison, 1994). Larvae of benthic invertebrate species predominantly require to settle and metamorphose in nearshore environments. Transport of larvae by currents to unsuitable non-benthic habitats offshore may also therefore result in death (Sebens, 1983; Scheltema, 1986).

### The Definition of competence

In order for the duration of the delay phase to be known, the onset of larval competence must be reliably determined. The exclusively behavioural criterion of larval ability to settle and successfully metamorphose has been employed to define

the onset of the larval competence (Doyle, 1975; Chia, 1978). However, the exact delay period undergone by a larva cannot be directly determined since the onset of competence can be diagnosed only by the successful induction of metamorphosis which is, of course, irreversible (Pechenik, 1984). The difficulties associated with obtaining an accurate definition of competence have been previously addressed in detail within Chapter Three.

### Examples of Delayed Metamorphosis

An ability to extend the pelagic period past the attainment of competence as been demonstrated within the laboratory for many marine invertebrate taxa, for example, among the bryozoans (Knight-Jones, 1953a; Nielson, 1981; Woollacott *et al.*, 1989), annelids (Williams, 1964; Doyle, 1975; Butman *et al.*, 1988; Pawlik, 1988; Pechenik & Cerruli, 1989), arthropods (Lucas *et al.*, 1979), urochordates (Olson, 1993), both asteroid (Knight-Jones, 1953a; Birkeland *et al.*, 1971; Strathmann, 1978; Nielson, 1981; Woollacott *et al.*, 1989) and echinoid echinoderms (Strathmann, 1978; Highsmith & Emlet, 1986; Rumrill, 1989), and bivalve molluscs (Bayne, 1965; Coon *et al.*, 1990; Pechenik *et al.*, 1990). In particular, there is a wealth of laboratory evidence documenting delay of metamorphosis among both prosobranch (Scheltema, 1961; Pechenik, 1980; Perron, 1981; Morse & Morse, 1984; Pechenik, 1984; Pechenik & Lima, 1984; Lima & Pechenik, 1985; Jaeckle & Manahan, 1989; Pechenik & Eyster 1989) and opisthobranch (Hadfield, 1977; Perron & Turner, 1977; Switzer-Dunlap, 1978; Chia & Koss, 1978; Kempf, 1981; Kempf & Hadfield, 1985, Paige, 1986, 1988; Miller, 1993) gastropods.

The ability to delay metamorphosis varies appreciably between species from a matter of hours to months (Pechenik 1990, Pechenik 1992). For example, 75% of the lecithotrophic larvae of the cheilostome bryozoan *Bugula stolonifera* attain competence within 3 h of release and can undergo a maximum delay of just 10-21 h before the ability to successfully metamorphose is lost. In contrast, some planktotrophic larvae may undergo extremely long pelagic periods whilst retaining the ability to metamorphose (Scheltema, 1971; Kempf, 1981). Such teleplanic (far-wandering) larvae originate from near-shore environments yet their long pelagic period may confer a high potential for dispersal (Scheltema, 1971; Kempf, 1981). Examples of species employing such larvae include members of the gastropod

family Cymatiidae, or hairy tritons (Strathmann, 1971). The planktotrophic larvae of the opisthobranch gastropod *Aplysia juliana* has been documented to possess the capacity to delay metamorphosis for a considerable period in the laboratory. Whilst competence may be attained 28 d post-hatching (Switzer-Dunlap & Hadfield, 1977) larvae have been documented to successfully delay metamorphosis for an additional 283 d (at 23.5 - 30.0°C)(Kempf, 1981).

### Larval Energetics

The factors determining the capacity of a species to delay metamorphosis have been addressed by many authors. The pelagic period was defined by Scheltema (1967) as consisting of the developmental phase, during which growth and differentiation took place, and the delay phase. In his study of the mud snail *Ilyanassa obsoleta*, Scheltema (1966) proposed that planktotrophic larvae entered an energetically balanced state during the extended competent phase, during which the net energy intake would meet only the basic metabolic demands, and provide no further energy for growth. The delay capacity of the larva would, therefore, be determined by the ability to achieve this state. Support for this hypothesis was inferred by the decrease in rate of shell growth observed in *I. obsoleta* during enforced delay of metamorphosis in the laboratory (Scheltema 1965, 1967). However, growth in *I. obsoleta* may not be accurately reflected by shell length. Indeed, a subsequent study both of shell length and larval organic weight in *I. obsoleta* by Pechenik (1980) established that larval mass increased at a constant rate during the delay phase, demonstrating that in this species an energetically steady state is not evident during the delay phase. In addition, several other marine invertebrate species have since been documented to display growth and/or differentiation during extended competent periods (Pechenik, 1984; Pechenik & Lima, 1984). In contrast to prosobranch gastropods, the attainment of competence among the Opisthobranchia is accompanied by a cessation of larval shell growth (Kempf & Willows, 1977; Chia & Koss, 1978; Kempf, 1981). For example, Kempf (1981) established that larvae of the aplysiid *Aplysia juliana* reached a state where both shell and tissue mass remained at a constant plateau throughout the extended competent phase, offering support that in some species an energetically steady state may indeed be attained during an extended pelagic period. Similar examples of cessation of larval growth on attainment of competence have been



reported for several other species of aplysiid opisthobranchs (Switzer-Dunlap & Hadfield, 1977) and nudibranchs (Kempf & Willows, 1977; Chia & Koss, 1978).

#### Selection for Length of Delay Phase

The larvae of marine invertebrates therefore show considerable inter-specific differences in both the precompetent and competent period. It was Pechenik (1980) and Johnson & Sutton (1981) who first linked the duration of these two components of the pelagic phase. It was noted that those species possessing short pre-competent periods tended to possess limited capacities to delay metamorphosis, whilst those with longer precompetent periods tended to exhibit considerably greater capacities for delay. The factors controlling the selection of such disparate inter-specific capacities to delay metamorphosis were examined by both authors in different manners. Pechenik (1980) examined the energetics and compared the delay capacity of three gastropods - *Ilyanassa obsoleta*, *Crepidula fornicata*, and *Bittium alternatum* - finding that, whilst *I. obsoleta* and *B. alternatum* could delay metamorphosis for 60d, *C. fornicata* could delay for only 30d. He also established morphological differentiation to occur during the delay phase both in *I. obsoleta* and *C. fornicata*, inferring an excess of energy to be available to the larvae above their basic metabolic requirements. This direct experimental evidence led Pechenik (1980) to postulate the pelagic phase to have a genetically programmed developmental sequence with a predetermined end-point. The length of the competent phase would therefore be proportional to the length of the precompetent phase whilst the absolute time period would be dependent upon the rate of growth and differentiation. Thus, the higher energetic efficiency demonstrated by *C. fornicata* larvae resulted in their developing more rapidly than both *I. obsoleta* and *B. alternatum*, and therefore displaying a shorter capacity to delay metamorphosis in comparison to the two other species under study. Subsequent studies on *C. fornicata* have also established the length of the delay phase to be affected by the rate of growth both of batch-cultured (Pechenik, 1984) and individually-reared larvae (Pechenik & Lima, 1984). Whilst the growth rates of individual larvae could be estimated only from entire larval cultures in Pechenik's (1984) original study, the subsequent paper of Pechenik & Lima (1984) monitored individual larvae and thus demonstrated the inverse relationship between rate of growth and the delay phase with a greater degree of certitude. This trend is not universal however; no

such link between rate of growth to competence and duration of the delay phase was observed by Bayne (1965) in his study of the bivalve *Mytilus edulis*.

In contrast to the experimental approach of Pechenik (1980), Johnson & Strathmann (1981) concluded the competent and precompetent period to be linked after examination of examples of delayed metamorphosis documented in the literature. Using the rationale that a long precompetent period will require a greater capacity to delay metamorphosis because of the increased probability of larval drifting from a suitable habitat, Johnson & Strathmann (1981) developed a model which incorporated offshore mixing and larval mortality. The model correctly predicted the trends observed in the literature, namely a positive correlation between length of precompetent and competent period and the competent period tending to be longer than, or at least equal to, the precompetent phase. The model was not universally successful however, and falsely predicted a decrease in the ratio of competent : precompetent period with increasing delay phase which was not apparent in the literature.

Therefore, whilst both Pechenik (1980) and Jackson & Strathmann (1981) agreed the delay capacity to be proportional to the precompetent phase the hypotheses presented for the selection of such differences in delay capacity are distinct. In contrast to Jackson & Strathmann (1981), Pechenik (1980) views the increase in dispersal capacity concomitant to an increased capacity to delay metamorphosis simply as a by-product of selection for another trait. Indeed, were it the case that the extended competent phase were selected to allow for drifting into unsuitable habitats caused by a long precompetent phase, as proposed by Jackson & Strathmann (1981), it might be expected that a shorter precompetent period would be selected for (Pechenik 1990).

Whilst the delay capacity of a larva may be a function of its developmental rate it appears also to be correlated with the degree of habitat specificity required by that species (Bayne, 1965; Crisp, 1974; Pechenik, 1980, 1985; Bickell & Kempf, 1983; Coon *et al.*, 1990). For example, oyster (*Crassostrea* sp.) larvae do not possess highly specific habitat requirements, and display the predicted limited capacity to delay metamorphosis (Coon *et al.*, 1990). In a similar manner, in his study of the delay capacity of *Crepidula fornicata*, *Ilyanassa obsoleta*, and *Bittium alternatum*, Pechenik (1980) concluded that *C. fornicata*, judged to have the lowest habitat specificity requirements of the three, also displayed the lowest capacity for delayed metamorphosis.



### Factors Affecting The Duration Of The Delay Phase

The capacity of a larva to extend the competent period may be affected by such environmental variables as water temperature, salinity, dissolved oxygen, pH and, in the case of larvae which feed, availability of food (see reviews of Thorson, 1950; Day & McEdward, 1984; Pechenik, 1985, 1987, 1990). Such external factors have the potential to alter the metabolic requirements of larvae, and therefore the rate at which energetic reserves will be utilized. Much research has focussed on the effect of environmental conditions on larval survival and growth (see Pechenik's [1987] review in particular). In contrast there is a relative paucity of literature concerning the effect of environmental variables on the duration of the delay phase. That literature which is available concerning the effect of environmental conditions on the duration of the delay phase has predominantly been supplied by Bayne (1965) for the bivalve *Mytilus edulis*, and by Pechenik and co-workers for the prosobranchs *Crepidula fornicata* and *C. plana*.

### The Influence of Temperature on the Duration of the Delay Phase

Water temperature is amongst the most important of environmental variables influencing marine organisms (Levington, 1982). There appears a clear trend towards decreasing larval development times with increasing temperature (Bayne, 1965; Pechenik, 1984 a & b; Pechenik & Lima, 1984; Zimmerman & Pechenik, 1991), although the rate will vary with the acclimation range of the organism (Landry, 1975). Larvae of *Mytilus edulis*, for example, raised at predetermined temperatures between 5 - 22°C, attained competence earlier at higher temperatures (Bayne, 1965). Concomitant with this shortened precompetent period was a reduction in the delay capacity of larvae: metamorphosis could be successfully delayed for 40d at 10°C, yet at 20°C this period decreased dramatically to just 2d. Reduced precompetent and total pelagic periods in response to raised temperature regimes have also been documented in the chiton *Mopalia muscosa* (Pechenik, 1984b) and gastropods *Crepidula plana* (Zimmerman & Pechenik, 1991) and *C. fornicata* (Pechenik, 1984a, Pechenik & Lima, 1984). Although larvae of *C. plana* were found to be smaller at attainment of competence at higher temperatures they also attained competence faster, indicating an uncoupling between mechanisms of

larval growth and larval differentiation (Zimmerman & Pechenik, 1991). Whilst the rate of growth of *C. plana* larvae subjected to higher temperatures did not increase, larvae did attain competence at an earlier age (Zimmerman & Pechenik, 1991). This reduction in precompetent period with increased temperature, indicative of an increased rate of tissue differentiation, was interpreted to establish that the process of larval growth (increase in size or mass) and tissue differential (temporal shifts in gene expression) were separate (Pechenik, 1984a; Zimmerman & Pechenik, 1991). The shorter duration of the precompetent and total pelagic periods observed for *M. edulis* (Bayne, 1965), *C. plana* (Pechenik, 1984a), *M. muscosa* (Pechenik, 1984b) and *C. fornicata* (Pechenik, 1984a; Pechenik & Lima, 1984) are consistent with the developmental model of larval life incorporating a predetermined end-point proposed by Pechenik (1980). The relationship between increased temperature and shortened larval period has been found to be non-linear (Byrne *et al.*, 1978; Pechenik, 1987; Zimmerman & Pechenik, 1991) and also to vary within the acclimation range of the organism (Landry, 1975). For example, the developmental rate of the mud snail *Ilyanassa obsoleta* was little affected by a rise of 3.5°C from 17.5°C to 21°C, yet was substantially increased by a further rise of 4°C (Scheltema, 1967).

In a similar fashion, salinity may affect the duration of the pelagic period (Bayne, 1965; Zimmerman & Pechenik, 1991). Larvae of *Mytilus edulis* from populations subjected to different salinity regimes were found to be less stressed and to possess a greater delay capacity within the 'normal' salinity range for that population (Bayne, 1965). Zimmerman & Pechenik (1991) established that *Crepidula plana* larvae displayed a shorter precompetent period when raised at 30‰ than at 25‰ and 20‰ and concluded salinity to have an effect on the duration of both the competent and precompetent periods.

#### The Influence of Food Availability on Duration of Delay Phase

The influence of food availability on the ability of invertebrate larvae to extend the competent period will be dependent upon larval type. Pelagic larvae rely, to differing extents, upon endogenous energetic reserves in the form of yolk and additional energy obtained externally through particulate feeding (Pechenik, 1987). Those larvae possessing an extended obligatory feeding stage - clarified by Thorson (1946) as planktotrophs - must assimilate sufficient particulate food to meet their

energetic requirements until metamorphosis to avoid starvation and death during the pelagic period (Pechenik, 1987). Planktotrophic larvae characteristically undergo longer precompetent and total pelagic periods relative to other larval types (Thorson, 1946; Strathmann, 1985; Pechenik, 1985; Todd, 1985; Havenhand, 1993) and therefore tend to display a greater capacity to delay metamorphosis (Jackson & Strathmann, 1981; Strathmann, 1985). Suboptimal food levels have been proposed to assert a range of effects on planktotrophic larvae, including increasing the developmental time, and thus total pelagic period (Thorson, 1946; Vance, 1973a & b). In the ocean planktotrophic larvae may be subject to periods of starvation resulting from patchiness in distribution of phytoplankton (Allison, 1994). Laboratory studies of the effect of temporary starvation on planktotrophic larvae have shown that not only is larval developmental rate slowed, and therefore the total pelagic phase extended, by periods of reduced food availability (Werhmann, 1991; Allison, 1994) but also by reduction in food quality (Werhmann, 1991; Pedrotti & Fenaux, 1993). Tolerance of food limitation, and therefore the effect of duration of the pelagic phase, also may vary both between larvae of differing ages (Allison, 1994) and between taxa; echinoderms, for example, generally appear less sensitive to periods of starvation than do crustaceans (Olson & Olson, 1989).

Assignment of larval feeding type has traditionally been determined by the presence or absence of particulate feeding during the pelagic phase (see Thorson, 1946). Those larvae which require to feed in the plankton during the pelagic phase in order to successfully metamorphose generally are termed planktotrophs, whereas those larvae which do not need to feed and instead rely solely on internal yolk reserves are termed lecithotrophs. Larvae now described as facultative planktotrophs were first referred to by Thorson (1946) as 'planktotrophic larvae with a short pelagic life'. These larvae, whilst possessing sufficient yolk reserves to successfully metamorphose, may also supplement these endogenous reserves by particulate feeding during the pelagic phase (Thorson, 1946; Hadfield, 1963, 1972; Chia, 1974; Perron, 1981; Kempf & Hadfield, 1985; Emlet, 1986b; Kempf & Todd, 1989). Facultative planktotrophy has been postulated to be an evolutionary intermediate stage in the shift from obligate feeding towards lecithotrophic development (Kempf & Hadfield, 1985; Kempf & Todd, 1989). The ability of facultative planktotrophs to supplement endogenous energy reserves has been proposed to allow an increased larval pelagic period (Hadfield, 1963; Perron, 1981;

Kempf & Hadfield, 1985; Miller, 1993; but see Emlet, 1986b), and will be further addressed within Section 5.4.

The traditional classification of larval type by the presence or absence of particulate feeding has not considered the uptake of exogenous nutrition in the form of dissolved organic material (DOM). Since the possible rôle of DOM in the nutrition of aquatic invertebrate larvae was first considered by Stephens & Schinske (1961), the uptake of DOM from the surrounding seawater medium has been demonstrated among echinoderm, molluscan and annelid larvae (Strathmann, 1975; Manahan *et al.* 1982, 1983; Pechenik, 1987; Manahan, 1990). Indeed, Strathmann (1975) concluded that DOM could constitute a significant source of external nutrition for echinoderm larvae. This development of larval ability to take up DOM has been proposed as a possible mechanism contributing to the supplementation of yolk reserves as species evolve from a planktotrophic towards a lecithotrophic mode of development (Kempf & Todd, 1989). Whilst many studies have exclusively concerned the uptake of dissolved amino acids (Stephen & Schinske, 1961; Manahan *et al.*, 1982, 1983), both the bivalve *Crassostrea gigas* and the gastropod *Haliotis rufescens* have been demonstrated to actively take up sugars from sea water (Wellborn & Manahan, 1990).

The absence of particulate feeding has led lecithotrophic, and indeed starved facultatively planktotrophic larvae, to be regarded as relying entirely upon internal energetic reserves (yolk) for sustenance until metamorphosis. Between hatching and the termination of the pelagic phase, therefore, lecithotrophic larvae would be expected to exhibit a net loss in mass. However, Jaeckle & Manahan (1989a) found the lecithotrophic larvae of *Haliotis rufescens* to show a decrease in weight over the larval period in only one of five larval cultures. The only other possible source of exogenous nutrition was postulated to be DOM, and indeed Jaeckle & Manahan (1989b) established that *H. rufescens* larvae were able to take up and to metabolise amino acids. An energy budget constructed by comparing the energy required for development with that provided by catabolism of energetic endogenous reserves, found that only 25 - 71% of larval energetic needs could be met by yolk reserves. This shortfall led Jaeckle & Manahan (1989a) to conclude that the larvae of this lecithotroph were 'feeding' by the uptake of dissolved organics from the surrounding sea water. This additional source of nutrition may be especially pertinent to lecithotrophic species by enhancing the larval ability to extend the duration of the competent phase.



The uptake of DOM certainly constitutes heterotrophic nutrition; that is, the acquisition of nourishment from exogenous organic material by organisms unable to synthesize organic compounds from inorganic substrates (Anon., 1993). Whether such nutrition by uptake of DOM may indeed be termed as 'feeding' is a subject for debate. Should traditionally defined non-(particulate) feeding lecithotrophic larvae, in which the uptake of nutrition in the form of DOM is established, now be termed facultative planktotrophs? Whilst Jaeckle & Manahan (1989 a, b) consider the uptake of DOM as 'feeding' (albeit not visible), Kempf & Todd (1989) considered that in those cases where DOM is used to supplement energetic reserves the term 'non-feeding development' should be applied in preference to 'lecithotrophic development'. Those larvae relying solely on endogenous energetic reserves (lecithotrophs) may, however, also be considered to undergo a form of 'non-feeding development'. Therefore, in applying these terms, no clear distinction in terminology exists between those larvae relying solely on endogenous energetic reserves and those supplementing those endogenous reserves by the uptake of DOM. Accepting the definition that food is 'a general term for any nutrient which is *taken in* or ingested by an organism and used by it to produce energy, build and repair body tissue, and regulate body processes' (Anon., 1991), it would seem appropriate to consider the uptake of DOM as a form of feeding. A modified definition of facultative planktotrophy may therefore be applied which will enable distinction to be made between particulate and dissolved sources of nutrition. For example, a lecithotrophic larvae such as that of *Haliotis rufescens*, which has been established to supplement yolk reserves solely by the uptake of DOM, could neither be described as a lecithotroph (as this term refers only to those larvae which rely solely on endogenous reserves), nor as a facultative planktotroph (as this term refers only to particulate feeding). More appropriate and precise terms to describe larval modes of nutrition may therefore be lecithotrophy, facultative *particulate planktotrophy* and facultative *DOM planktotrophy*.

#### Field Evidence For The Occurrence Of Delayed Metamorphosis

In contrast to the wealth of literature documenting delayed metamorphosis in the laboratory, there is a relative paucity of literature presenting evidence for the occurrence of delayed metamorphosis in the field. Determination of the delay phase *in situ* is fraught with the logistical difficulties attendant with tracking such a small target, including restricted visibility (and therefore identification) of the subject and

the relatively long duration of both the precompetent and competent periods (Pechenik 1990). Evidence that delayed metamorphosis does indeed occur in the field, and may thus constitute an ecologically significant phenomenon has therefore been obtained predominantly by inference. For example, competent larvae of the hemichordate *Ptychodera flava* were found by Hadfield (1978) to be present in plankton tows around Hawaii from mid-February to early September, although young are produced only during the restricted spawning season from mid-November to late December. Shock-induced initiation of metamorphosis on collection from the plankton enabled Hadfield to establish that competence was obtained within four months of spawning. Accepting the assumption that larval provenance was exclusively local, the occurrence of competent larvae outwith the spawning season led Hadfield to conclude *P. flava* to be capable of delaying metamorphosis for at least four months in the field.

Plankton tows have also established the presence of gastropod larvae originating from near-shore habitats in both the mid-Atlantic and Pacific oceans, intimating such larvae to have the capacity to undergo considerable pelagic periods (Scheltema, 1971, 1986b). Using the velocity of ocean currents Scheltema (1971) estimated the total pelagic period of ten prosobranch gastropods found in the mid-Atlantic. Comparison with known precompetent periods led Scheltema (1971) to conclude that four of these species displayed considerable delay phases. Considering the impact of this capacity on genetic differentiation of populations on the east and west coasts of the Atlantic, he found that species displaying considerable capacities for metamorphic delay did show the predicted low degree of morphological differentiation between populations. Scheltema (1971) interpreted this as intimating that the capacity to delay metamorphosis had fundamentally affected population structure in these species. In the absence of genetic data it is not possible to determine whether this low degree of morphological differentiation is indeed a reflection of genotype or whether it is merely phenotypic.

An alternative approach has been to compare the morphologies of larvae from the field with laboratory reared larvae of known age. Whilst any comparison of larvae of unknown provenance and history with laboratory-reared individuals must be drawn with caution, this approach has proved useful in indicating the capacity to delay metamorphosis in many invertebrate species (Bayne, 1965; Domanski, 1984; Emlet, 1986; Pechenik, 1986). For example, Emlet (1986b) examined the larval morphology of the echinoid *Dendraster excentricus* taken from

a shallow fjord in Puget Sound, Washington. Using the findings of Highsmith & Emlet (1986), that larval arm rod length decreased with increasing delay phase in this species, Emlet (1986b) concluded the field-collected individuals to have delayed metamorphosis for at least several weeks. Similarly, larvae of the asteroid *Luidia sarsi* may settle and metamorphose (lose the larval body) when the developing sea-star body diameter reaches 3.5 mm (Domanski, 1984). Examination of larvae obtained from the Porcupine Sea Bight in the N. Atlantic by Domanski (1984) revealed unmetamorphosed larvae with sea-star body diameters of up to 11 mm, which (assuming a constant growth rate) was estimated to be consistent with a total planktonic phase of over a year.

The comparative morphology of field obtained and laboratory raised larvae has also been employed to provide support for the occurrence of delayed metamorphosis in members of the Mollusca, both for the bivalve *Mytilus edulis* (Bayne, 1965), and gastropods *Crepidula plana*, *C. fornicata*, and *Bittium alternatum* (Pechenik, 1986). After attaining competence the planktonic larvae of both *C. plana* and *C. fornicata* continue to increase in shell dimension, and attain a visible shell brim. Thus, Pechenik (1986) was able to discriminate between the precompetent larvae present in the plankton and those undergoing an extended competent period. Whilst all three gastropod species under examination did indeed show evidence of delayed metamorphosis in the field, the unknown nutritional status of field collected larvae precluded estimates of delay periods based on larval shell size.

A rare example of an *in situ* delay phase obtained by direct observation is provided by the tropical colonial ascidian *Lissoclinum patella* (Olson & McPherson, 1987). Successful tracking of larvae was facilitated by both the relatively short pelagic period (2 h) and the large size of the larvae (5.5 mm) in addition to the shallow distribution of *L. patella* settlement sites (<10 m depth). Larvae were monitored *in situ* either confined within a closed box or swimming freely. Whilst confined larvae swam for more than two hours, unconfined larvae swam for less than ten minutes. The difference in observed larval behaviour was estimated to reduce the effective dispersal range from several hundred m to less than ten m. The disparate pelagic periods and consequent dispersal potentials within this study illustrate the caution which must be exercised when considering the accuracy, and ecological significance, of laboratory derived delay periods.



### The Costs of Delayed Metamorphosis

The ability to extend the competent phase may be of adaptive value only if larvae, having undergone a delay phase, then successfully metamorphose (Day & McEdward, 1984; Pechenik, 1986). There are numerous examples cited previously within this section of invertebrate species possessing the capacity to successfully delay metamorphosis. The duration of the delay phase is however, finite (Pechenik, 1984, 1985, 1987), and larval energy reserves may be depleted by an extended delay phase (Miller 1993). An extension of the delay period beyond a critical point may therefore result in unsuccessful metamorphosis in addition to exerting more subtle deleterious effects on those larvae which may successfully metamorphose (Lucas *et al.*, 1979; Nielson, 1981; Sebens, 1983; Pechenik, 1985, Highsmith & Emlet, 1986; Pechenik & Cerulli, 1989; Woollacott *et al.*, 1989; Miller, 1993). The consequences of an extended delay period upon subsequent life history may for example include reduced metamorphic success (Lucas *et al.*, 1979; Miller, 1993) and juvenile survival and growth rate (Nielson, 1981; Highsmith & Emlet, 1986; Pechenik, 1989; Woollacott *et al.*, 1989). The effects of an extended delay phase on subsequent selected life history parameters are presented in Table 5.1, and will be addressed in detail in Section 5.4. An increase in delay phase may also lead to decreased cue specificity which might itself result in reduced fitness through the recruitment of larvae to suboptimal habitats (Knight-Jones 1953, Sebens 1983).

### Rationale

1. *Adalaria proxima* has been reported to successfully delay metamorphosis for between 10 d (Todd & Havenhand, 1985) and 14 d (Thompson, 1958), although Kempf & Todd (1989) estimated a possible maximum delay capacity of 15 - 20 d. The initial objective of this study was therefore to obtain a more exact indication of the delay capacity of *A. proxima* veliger larvae.

2. The deleterious effects of an extended competent period both on larval and juvenile survival and growth have been previously described (Lucas *et al.*, 1979; Nielson, 1981; Highsmith & Emlet, 1986; Pechenik & Cerruli, 1989; Woollacott *et al.*, 1989). For example, an increased pelagic phase imposed upon the lecithotrophic larvae of the nudibranch *Phestilla sibogae* resulted in lower larval and post-larval weights, decreased survival and post-metamorphic success in addition to

**Table 5.1. The Effects of an Extended Delay Phase on Juvenile Survival and Growth Rate.** Dashed lines (-) denote no data available.

Species	Larval Type	Duration of Delay Phase (d)	Temp (°C)	Juvenile Survival	Juvenile Growth Rate	Authority
<b>Gastropods</b>						
<i>Aplysia juliana</i>	Planktotroph	283	23-30	-	Not Affected	Kempf (1981)
<i>Crepidula fornicata</i>	Facultative Planktotroph (fed)	<6	25	Not Affected	Not Affected	Pechenik & Eyster (1989)
<i>Phestilla sibogae</i>	Facultative Planktotroph (fed)	7 - 28	26	Not Affected	Not Affected	Miller (1993)
	(unfed)	7 - 28	26	Not Affected	Not Affected	Miller (1993)
<b>Polychaete</b>						
<i>Capitella</i> sp.I	Lecithotroph	5	15	Not Affected	-	Butman <i>et al.</i> (1988)
<i>Capitella</i> sp.I	Lecithotroph	5	20	Decreased	Not Affected	Pechenik & Cerruli (1991)
<b>Echinoderms</b>						
<i>Dendraster excentricus</i>	Planktotroph	42	8 - 10	Decreased	Not Affected	Highsmith & Emlet (1986)
<i>Strongylocentrotus droebachiensis</i>	Planktotroph	28	-	Not Affected	Not Affected	Highsmith & Emlet (1986)
<i>S. droebachiensis</i>	Planktotroph	7 - 52	-	Not Affected	Not Affected	Rumrill (1989)
<i>Echinorachnius parma</i>	Planktotroph	28	10- 11	-	Not Affected	Highsmith & Emlet (1986)
<b>Arthropod</b>						
<i>Semibalanus balanoides</i>	Planktotroph	14	10	Not Affected	-	Knight-Jones (1953a)
<b>Bryozoans</b>						
<i>Hippodiplosia insculpta</i>	Lecithotroph	2 h	16-21	-	Decreased	Neilson (1981)
<i>Bugula stolonifera</i>	Lecithotroph	5-7 h	20	-	Decreased	Woollacott <i>et al.</i> (1989)

a lower and later reproductive output (Miller 1993). The lecithotrophic larvae of *A. proxima* also have the capacity to survive an extended competent phase, but the effect of metamorphic delay on larvae and post-settlement juveniles is unknown. The objective of this section therefore was to determine the presence and severity of any effects resulting from an extended delay phase on subsequent larval life-history parameters. This was performed by monitoring larval survival and the components of mortality, metamorphic success, and the size of newly metamorphosed juveniles in response to set periods of enforced delay.

3. It was Thompson (1958) who first observed particulate feeding in the veliger larvae of *A. proxima*. Whilst he successfully reared larvae on the phytoplankters *Chlorella stigmatophora* and *Isochrysis galbana*, he concluded that feeding was of little functional importance in this species because metamorphosis could be successfully attained in the absence of food. A subsequent comprehensive investigation into larval feeding in *A. proxima* by Kempf & Todd (1989) confirmed the status of *A. proxima* as a facultative planktotroph. Both fed and starved larvae were subjected to a 10 d enforced delay phase and were found to lose energetic content (J) during the course of the study, although fed larvae lost significantly less than did starved larvae. However, this did not appear to correlate with larval delay capacity : Kempf & Todd (1989) found that nutritional status had no influence on larval longevity, although they did propose that the ability of facultatively planktotrophic larvae to feed may be of importance in enhancing capacity to delay metamorphosis. The maximum delay period employed by Kempf & Todd (1989) was 10 d, and whilst the effect on larval biochemistry of facultative planktotrophic feeding was extensively covered, little attention focussed on the effect of such feeding on subsequent larval life-history parameters. This present study was consequently designed to establish whether developmental mode (e.g. imposed lecithotrophy or facultative planktotrophy) influenced the effects of an extended delay phase.

## 5.2 METHODS

### Collection and Maintenance of Adults and Spawn Masses

Experiments were performed over two annual spawning seasons. Juvenile *A. proxima* were collected from Menai Bridge (Anglesey), Loch Eriboll (Sutherland) and adult *A. proxima* were collected Kinkell Braes (Fife) and Clachan Seil (Argyll) during February - May 1993 and 1994. Juveniles, adults and resultant spawn masses were maintained within the laboratory at 10°C as previously described in Chapter 3 (Section 3.2). Juveniles and adults were supplied with excess food (*Electra pilosa*) collected from Kinkell Braes, St. Andrews, and Kingsbarns, Fife. Tanks were monitored for newly spawned masses daily. Spawn masses were immediately removed from parent tanks, isolated into china crucibles and cultured at 10°C with the entire water volume (25 ml) changed daily.

### Larval Culture

Spawn mass development was monitored using a Wild-E binocular microscope. On hatching, veligers were transferred to autoclaved (115°C for 15 min) Pyrex glass culture vessels containing 800 ml of twice filtered (0.45µm, thence 0.2µm) sea water, hereafter referred to as TFSW. Cultures were covered with Parafilm<sup>®</sup> and maintained at 10°C. To prevent bacterial and protozoan infection larval cultures were treated with an antibiotic mix to yield final concentrations of 60 mg l<sup>-1</sup> Penicillin-G (Benzylpenicillin, Sigma Chemical Co.) and 50 mg l<sup>-1</sup> Streptomycin Sulphate (Sigma Chemical Co.). No mixing of progeny between different spawn masses was allowed, and each culture vessel therefore contained only sibling cohorts of larvae. All glassware and equipment was hot-washed without detergents and was autoclaved before use.

The entire volume of culture water (800 ml) was changed every 4 d. Veliger larvae required to be concentrated before *en masse* transference to a clean culture beaker. This was achieved using a 250ml 'Tripour' beaker modified by replacing the beaker bottom with 125µm aperture Nitex<sup>®</sup> mesh gauze (affixed with Araldite non-toxic glue). The resulting 125µm filter was then held level within a glass

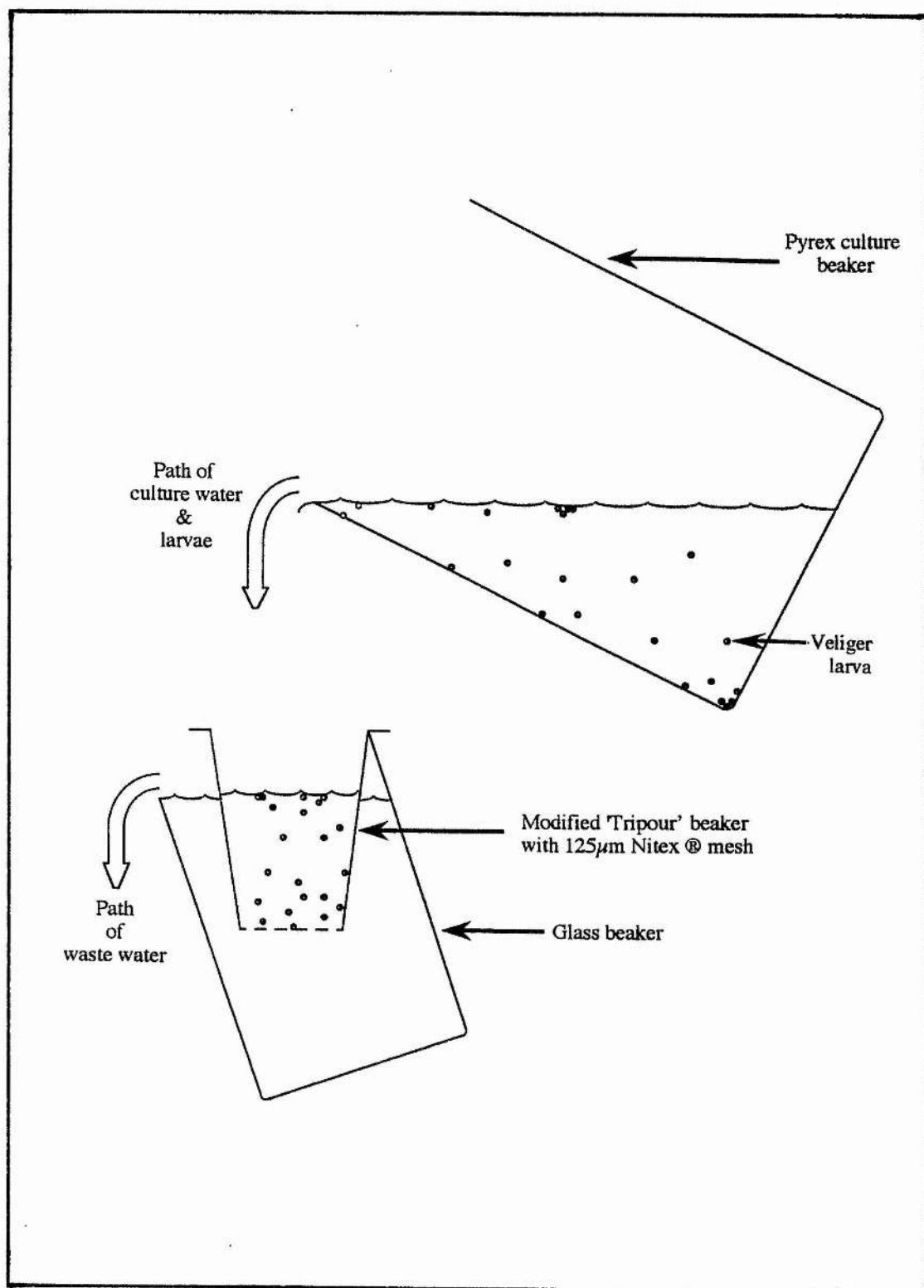
beaker of comparable size at an angle of 45° from the vertical to ensure constant immersion of larvae during the filtering process (see Plate 5.1 for diagram of apparatus). The larval culture to be changed was then poured slowly through the 'Tripour' filter assembly, whilst ensuring that the 'Tripour' handles rested above of, and the water level was below, the top of the glass beaker at all times. Both beaker and filter were then rested upon a light box and larvae again concentrated (to facilitate transference) by rotating the 'Tripour' filter within the stationary beaker. The resultant vortex transferred veligers into the central portion of the gauze from where they could be gently transported into the fresh culture container using a Pasteur pipette. The hydrophobic nature of the larval shell frequently resulted in *A. proxima* veligers becoming entrapped at the air/water interface, and subsequently 'rafting' into groups on the surface (see Plates 5.2a & b). In addition, the velar cilia of trapped larvae were observed to become clogged by mucus, compromising their ability to feed. This undesirable artefact of culture conditions was therefore minimized by placing cultures on a light box and 'sinking' entrapped larvae using drops of TFSW from a Pasteur pipette held above the water surface.

### Algal Culture

Larval cultures were raised as either facultative planktotrophs (fed conditions) or as lecithotrophs (unfed conditions). The length of the maximum delay period resulted in a considerable reduction in larval numbers (due both to mortality and to subsampling) by Day +28. Cultures therefore required high initial numbers of veligers, in order that sufficient numbers be present for sampling at the maximum delay period. Thus, whilst splitting each spawn mass into separate fed and starved cultures may have facilitated the study of larval nutritional status and delay phase, this was possible for only two exceptionally large spawn masses. A unialgal diet was supplied to the facultative planktotrophic treatments by the addition of the naked flagellate *Rhodomonas* sp., the stock culture of which was obtained from The Culture Collection of Algae & Protozoans, Plymouth Marine Laboratory. Algal batch cultures were cultivated in Provasoli's E. S. Medium, details of the preparation and composition of which are presented in the Appendix to Section 5.2. (adapted from Provasoli, 1968). Triplicate batches were set-up at 5 d intervals under conditions of constant illumination and aeration at 26 - 28 °C. The status of larval cultures was monitored both by examination of subsamples under an Olympus - CH compound microscope and by culture colour. Algae were

**Plate 5.1. Diagram of Larval Culture Filtering Apparatus.** Both fed and unfed larval cultures were transferred to sterile Pyrex culture beakers containing TFSW with antibiotics every 4 d (see text for details). Larvae were routinely filtered and concentrated for transference to the clean culture beakers using the filtration apparatus illustrated here.





**Plate 5.2a. (above) An Extensive Aggregation of 'Rafted' Veliger Larvae of *Adalaria proxima*.** The hydrophobicity of veliger shells frequently results in aggregations of larvae which have become trapped at the air/water interface. Whilst velar lobes (VL) remain extended, feeding may be compromised by clogging of velar cilia with mucus. Scale bar represents 200µm.

Additional abbreviation used in the plate shows:

ELS    evacuated larval shell

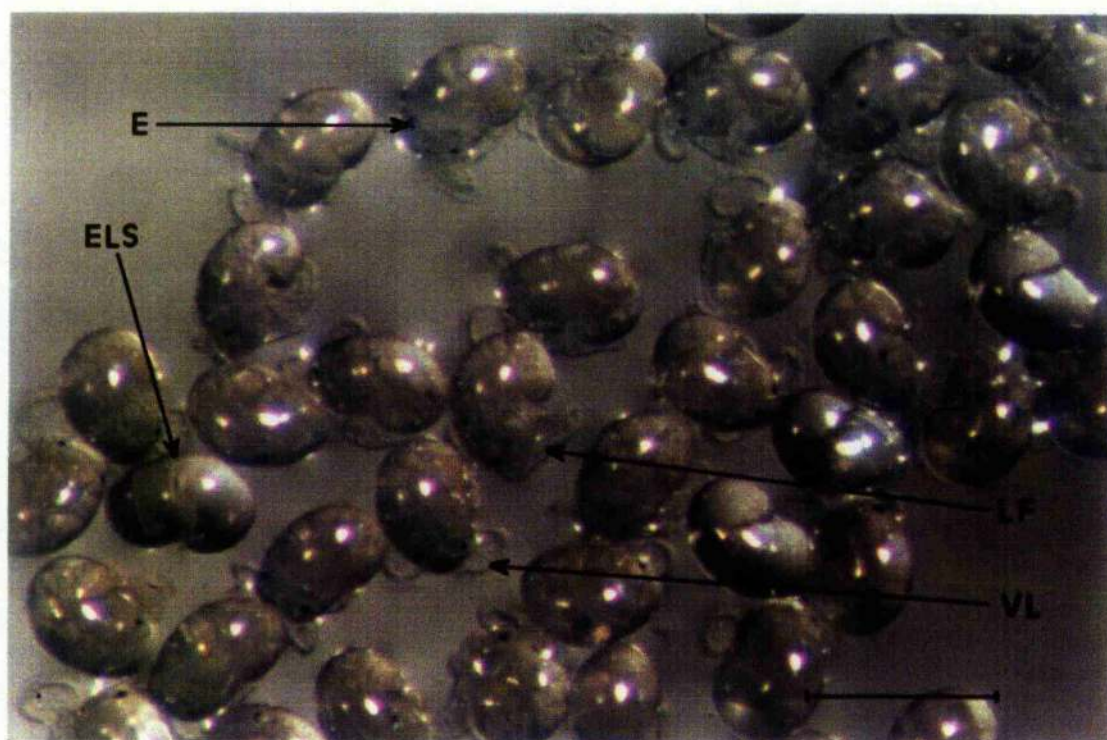
**Plate 5.2b. (below) Aggregation of 'Rafted' Veligers.** Both the extended velar lobes (VL) and colouration of the larval gut illustrated in this plate indicate that larvae may continue to feed whilst trapped . That the veliger remains at the surface solely due to the hydrophobic nature of the shell is evidenced by the presence of evacuated larval shells (ELS) at the air-water interface. Evacuation of the shell (due to metamorphosis or spontaneous evacuation) therefore allows the metamorph or spontaneous evacuee to descend back into the water column leaving the empty shell behind upon the surface.

Scale bar represents 200µm.

Additional abbreviations used in plate show:

E       larval eye

LF      larval foot



harvested only from healthy cultures which were actively growing (i.e. in the log-phase of growth).

Harvesting of algae for use as a planktotrophic food source was performed by centrifuging (2,500 r.p.m. for 10 min at 10°C) subsamples taken from the main batch cultures of algae. The resulting supernatant was then discarded, the algal pellet resuspended by shaking in TFSW and centrifugation repeated. Algal cell concentration (cells·ml<sup>-1</sup>) was then determined under an Olympus-CH compound microscope using a haemocytometer. All resulting cell densities were calculated from the means of quintuplicate counts. The algal density was then manipulated by dilution to a constant of 4·10<sup>-7</sup> cells·800ml<sup>-1</sup>.

### Experimental Protocol

The onset of competence in *A. proxima* has been documented to occur between 24-48 hr post-hatching (Thompson, 1958; Todd *et al.*, 1991) and has been previously discussed within Chapter 3. Subsamples of larvae were removed from cultures at 4-8 d, 21 d and 28 d post hatching, and induced to metamorphose by immersion in 5·10<sup>-3</sup> mM choline chloride solution. A mean of 41 larvae were taken per subsample, although the size of subsamples was dependent upon both the number of larvae in the larval culture and the age of the culture, resulting in numbers of subsampled larvae ranging between a minimum of nine and a maximum of 165. The choline solution was prepared as previously described in both Sections 3.2 and 4.2. Subsamples of larvae were placed in autoclaved covered glass petri dishes (55 mm diameter, 20 mm high) containing 15 - 20 ml choline chloride and incubated at 10°C. Choline was chosen to induce metamorphosis in preference to the natural cue because of the considerable degree of variability in metamorphosis observed in response to the natural cue during the course of concurrent investigations (see Chapter 4). Any such inconsistency in effect was considered to potentially hinder the interpretation of any age related effects on metamorphic success, and therefore choline chloride, which had displayed more consistent morphogenic properties, was used.



### Larval mortality and Metamorphic Success

Choline treated subsamples were inspected under a Wild-E binocular microscope 2 d after immersion in choline. The same criterion were employed throughout this study (see Chapters 3 & 4) to score subjects as pediveligers, spontaneous evacuees or successful metamorphs. In addition, the number of dead pediveligers, spontaneous evacuees and metamorphs were scored for each set delay phase.

### Juvenile Size, Growth, and Survival

Juvenile size was determined with the use of a Wild-E binocular microscope fitted with an ocular micrometer accurate to within  $4.47\mu\text{m}$ . Whilst *Adalaria proxima* metamorphs possess no hard fixed anatomical feature such as a shell, size was determined by measuring the maximum mantle length of newly metamorphosed crawling juveniles. Under observation under the binocular microscope the mantle length of individual metamorphs appeared to remain constant during crawling. The relationship between mantle length to width among 100 newly metamorphosed juveniles from a total of five spawn masses was examined to determine the validity of using mantle length as a non-destructive indication of metamorph size. The measurement of mantle length is demonstrated in Plate 5.3. Those juveniles on their sides or which were contorting their body shape were excluded from measurement.

### Photomicroscopy

Plates 5.2 a & b were obtained using a Wild-E binocular microscope fitted with an Olympus camera attachment. Larvae were held within petri dishes and illuminated by incident light provided by a cold light source.

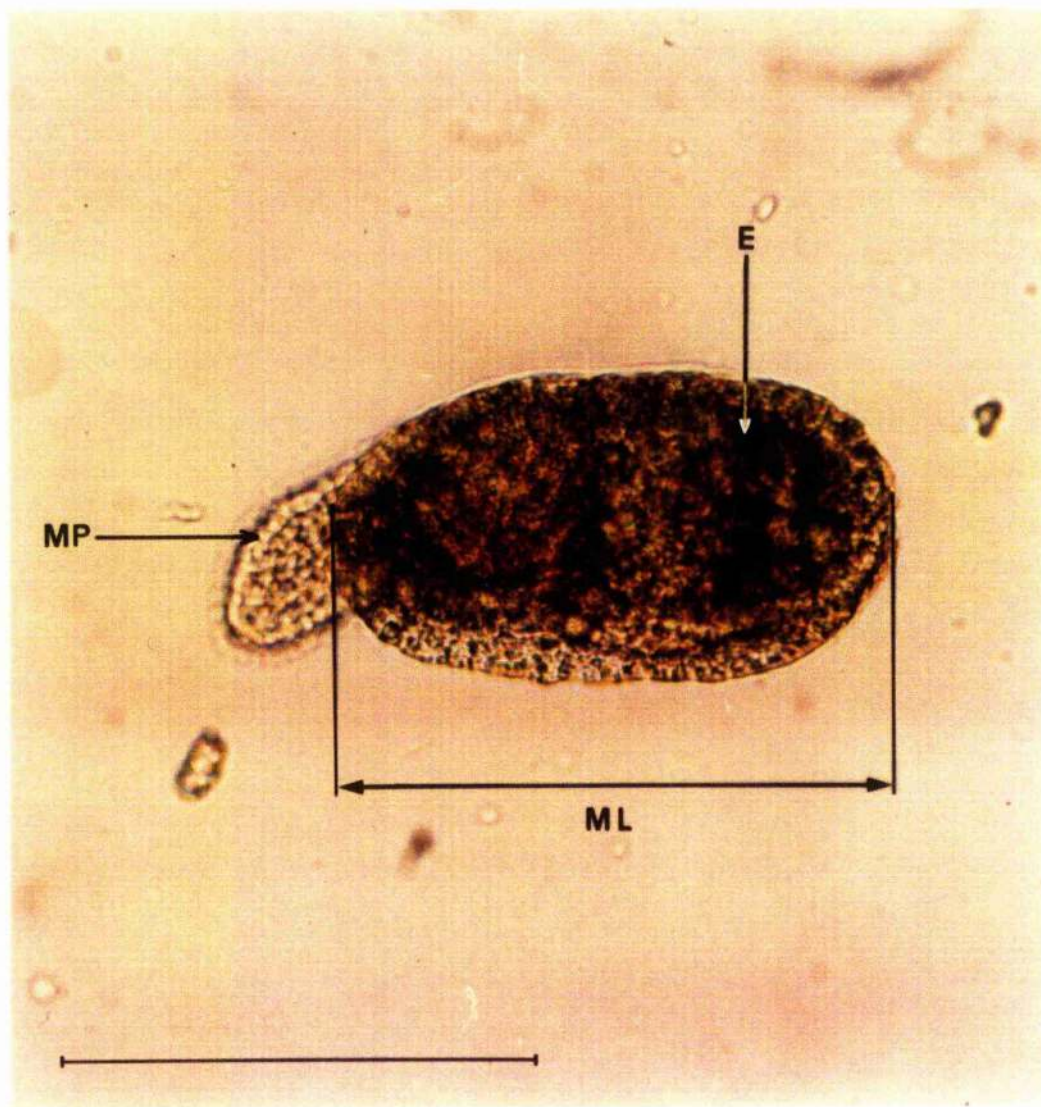
The photomicrograph presented in Plate 5.3 was obtained using a Leitz Diaplan microscope with a Wild Photoautomat MP545 attachment. The subjects were held within petri dishes and anaesthetized by the gradual addition of 15% magnesium chloride in TFSW. After examination under a Wild-E binocular

**Plate 5.3. Photomicrograph of benthic juvenile showing measurement of maximum mantle length: dorsal aspect.** Mantle length was determined with the use of a Wild-E binocular microscope fitted with an optical micrometer. Only the mantle lengths of healthy juveniles that were not contorting their shape at the time of measurement were noted. The length of the metapodium (seen here extending from the rear of the mantle) was observed to change constantly and was not included in the measurement. Scale bar represents 200 $\mu$ m.

Additional abbreviations used in plate show:

MP    metapodium

E     eye





microscope specimens were quickly transferred to a concave-welled microscope slide for photomicroscopy.

### Data Analysis

Data analysis was performed using the Minitab 8.1 Statistical Software Package. Larval mortality and metamorphic responses were converted to percentages and thereafter normalized by arc-sine transformation for all statistical analysis. The relationship between metamorph mantle length and width was examined using simple linear regression by least squares to obtain the relevant coefficient of determination. Factorial analysis was performed upon the data to test for any interaction in effect between developmental mode (facultatively planktotrophic or lecithotrophic) and duration of delay phase on any given response variable. The Minitab 8.1 Statistical Software Package is able to perform Model I two-way analysis of variance (ANOVA) only on balanced data sets. The use of eleven unfed and five fed cultures within this section therefore necessitated factorial analysis by Minitab using a general linear model (GLM). The simple two-factor crossed GLM model used was  $Y = A + B + A*B$  (where Y represents the response variable, and A and B the fixed factors). Subsequent one-way ANOVAs between duration of delay phase and selected response variables were then performed within each development mode separately. Where delay phase was established to have a significant effect on a variable, a subsequent Tukey's HSD was performed to identify the significant differences in treatment means. The resulting Tukey groupings were considered significantly different at the  $P < 0.05$  level and are expressed as superscript letters adjacent to the relevant datum. Data are expressed in Figures 5.1-5.4 and Tables 5.2a. and 5.4 as back-transformed means (in percentage form), and, where appropriate, are displayed within figures with appropriate standard error bars.

### 5.3 RESULTS

#### Metamorphic Success

Examination of the effect of delay phase and nutrition on metamorphic success by two-way ANOVA (using GLM, see Table 5.2b.) revealed that whilst an enforced extension of larval pelagic period resulted in decreased metamorphic success ( $P < 0.001$ ), larval nutritional state (facultatively planktotrophic or lecithotrophic) had no significant effect on the metamorphic success of resulting larvae ( $P = 0.442$ ). The metamorphic success of lecithotrophic larvae decreased significantly ( $P < 0.001$ ) from a maximum of 74.3% at the beginning of the delay phase down to just 35.4% at the termination of the experiment on Day +28 (see Table 5.2a and Figure 5.1). A *posteriori* multiple comparison by Tukey's HSD (also presented in Table 5.2a) revealed that lecithotrophic larval success dropped significantly within the first 21 days of the delay period. Whilst metamorphic success continued to decrease thereafter - from 49.9% after a 21 day pelagic period down to 35.4% after a 28 day delay - this was not considered significant at the  $P < 0.05$  level. The metamorphic success of facultatively planktotrophic larvae also steadily decreased with increasing delay phase (see Table 5.2a) from an initial 63.6% down to 28.2% in response to the maximum delay phase of 28 days. In contrast to the metamorphic success exhibited by lecithotrophic larvae however, this decrease did not constitute a significant drop when subjected to one-way ANOVA ( $P = 0.066$ ). The effects of an extended pelagic period on the metamorphic success of both facultatively planktotrophic (fed) and lecithotrophic (unfed) *Adalaria proxima* larvae are illustrated by Figure 5.1. The lack of a significant interactive term in the two-way ANOVA (by GLM) presented in Table 5.2b indicated there to be no significant difference in the metamorphic success exhibited between facultatively planktotrophic and lecithotrophic larvae in response to a delay in metamorphosis ( $P = 0.611$ ).

#### Total Mortality

The measurement of total mortality was by summation of the three discrete classes of mortality data - those of veliger, spontaneous evacuee and metamorph

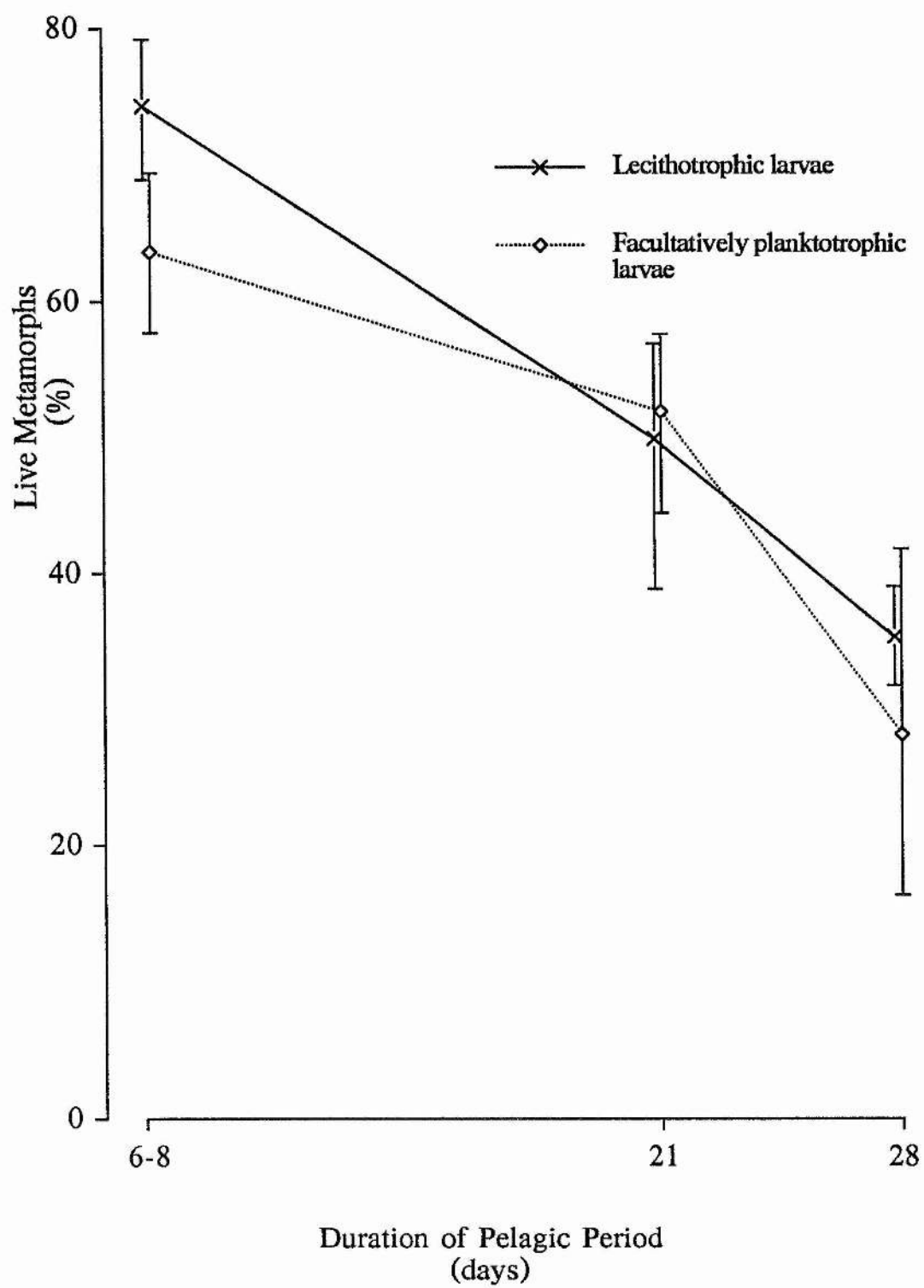
**Table 5.2a. (above) The Effect of an Extended Delay Phase on the Metamorphic Success both of Fed (Facultatively Planktotrophic) and Unfed (Lecithotrophic) *A. proxima* larvae.** Metamorphic success was defined as the proportion of live metamorphs (expressed in percentage terms) scored 2 days after administration of a known metamorphic cue ( $5 \cdot 10^{-3}$ M choline chloride). Data are the back-transformed means and show that the proportion of successful metamorphs appears to decrease with increasing delay phase in both nutritional treatments. Tukey Groupings resulting from analysis of the arc-sine transformed data are expressed within the table as superscript letters adjacent to the relevant mortality levels and denote those metamorphic responses considered significantly different at the  $P < 0.05$  level.

**Table 5.2b. (below). Factorial Analysis of the Effects both of Duration of Delay Phase and Nutritional State (facultatively planktotrophic or lecithotrophic) on the Metamorphic Success of *Adalaria proxima*.** Presented are the results of two-way ANOVA by GLM (general linear model) and subsequent one-way ANOVAs performed separately for the effects of delay phase on the metamorphic success of both lecithotrophic and facultatively planktotrophic larvae. Asterisks denote those responses considered significant at the  $P < 0.05$  level. It is evident that whilst the duration of the delay phase exerts a significant effect upon metamorphic success ( $P < 0.001$ ), nutritional state does not ( $P = 0.442$ ). Further, the nonsignificant interactive term ( $P = 0.611$ ) would indicate nutritional state not to influence the effect that duration of delay phase has on metamorphic success. One-way ANOVAs establish that whilst both metamorphic success appears to decrease with increasing delay phase (see Table 5.2a above), only among facultatively planktotrophic larvae is this decrease considered to be significant ( $P < 0.001$ ).

Nutritional Status	Metamorphic Success (%)			
	n	4-8 days	21 days	28 days
Facultative Planktotrophic Larvae	5	63.6	51.9	28.2
Lecithotrophic Larvae	11	74.3 <sup>a</sup>	49.9 <sup>b</sup>	35.4 <sup>b</sup>

	df	SS	MS	F value	P
<i>Results of Two-way ANOVA by GLM</i>					
Duration of Pelagic Phase	2	4007.5	1641.0	10.6	<0.001*
Nutritional State	1	93.4	93.4	0.60	0.442
Interaction	2	154.2	77.1	0.50	0.611
<i>Results of separate one-way ANOVAs</i>					
Lecithotrophic larvae					
among delay periods	2	3048	1524	10.12	<0.001*
within delay periods	30	4518	151		
Facultatively planktotrophic larvae					
among delay periods	2	1127	564	3.44	0.066
within delay periods	12	1964	164		

**Figure 5.1. The Effect of Extended Pelagic Period on the Metamorphic Success both of Facultatively Planktotrophic (fed) and Lecithotrophic (unfed) Larvae.** Metamorphic success was practically defined as the proportion of live metamorphs (in percentage terms) present two days after administration of a known artificial metamorphic inducer ( $5 \cdot 10^{-3}M$  choline chloride). Data are back-transformed means (expressed in percentage terms) obtained from subsamples of eleven larval cultures (lecithotrophic larvae) and five cultures (facultatively planktotrophic larvae). The corresponding absolute values of metamorphic success are displayed in Table 5.2a.



mortality. Factorial analysis of the effect of both delay phase and larval nutritional state (facultatively planktotrophic or lecithotrophic) on total mortality is presented in Table 5.3. An extension of larval pelagic period resulted in an increase in subsample mortality ( $P < 0.001$ ) when presented with an appropriate metamorphic stimulus (the artificial metamorphic induction agent choline chloride). In a similar trend to that established for metamorphic success (see above), larval nutritional state had no effect on total mortality ( $P = 0.440$ ) nor did it influence the effect of an extension in pelagic period on total mortality ( $P = 0.550$ ). The effect of an extended pelagic period on the mortality both of facultatively planktotrophic and lecithotrophic larvae is illustrated by Figure 5.2. Separate ANOVAs performed upon the mortality data for both nutritional states showed that whilst a trend of increasing total mortality was evident for facultatively planktotrophic and lecithotrophic larvae, this increase in mortality could be considered significant only among lecithotrophic (unfed) larval cultures ( $P = 0.001$ , see Table 5.4a.). The back-transformed mean mortality of lecithotrophic subsamples rose from a minimum of 13.9% after a 4-8 day delay to 41.4% after 21 days, and up to a maximum value of 64.0% after a 28 day delay (see Table 5.4a.).

#### The Constituent Components of Total Mortality

Factorial analyses designed to examine the effect of both an extension of the competent period and of larval nutritional state were performed for each class of mortality (the results of which are presented in Table 5.3). The veliger mortality data for facultatively planktotrophic veliger larvae (see Tables 5.4a & b) clearly shows no trend over time ( $P < 0.637$ ), mortality figures being 14.4%, 7.1% and 13.7% for 4-8 days, 21 days and 28 day pelagic periods respectively. In contrast, Table 5.4a. appears to show a trend of increasing mortality among lecithotrophic veligers, from 4.8% for the initial delay period increasing to 7.8% and 13.9% after 21 and 28 days respectively, although analysis by one-way ANOVA (presented in Table 5.4b) revealed this increase to be nonsignificant ( $P = 0.201$ ). Indeed, neither duration of the pelagic phase, nor larval nutritional state were shown to have a significant effect on the mortality exhibited by veligers ( $P = 0.416$  and  $P = 0.441$  respectively, see Table 5.3). Further, no difference was established between the levels of veliger mortality exhibited by lecithotrophic and facultatively planktotrophic larvae ( $P = 0.447$ , see Table 5.3).

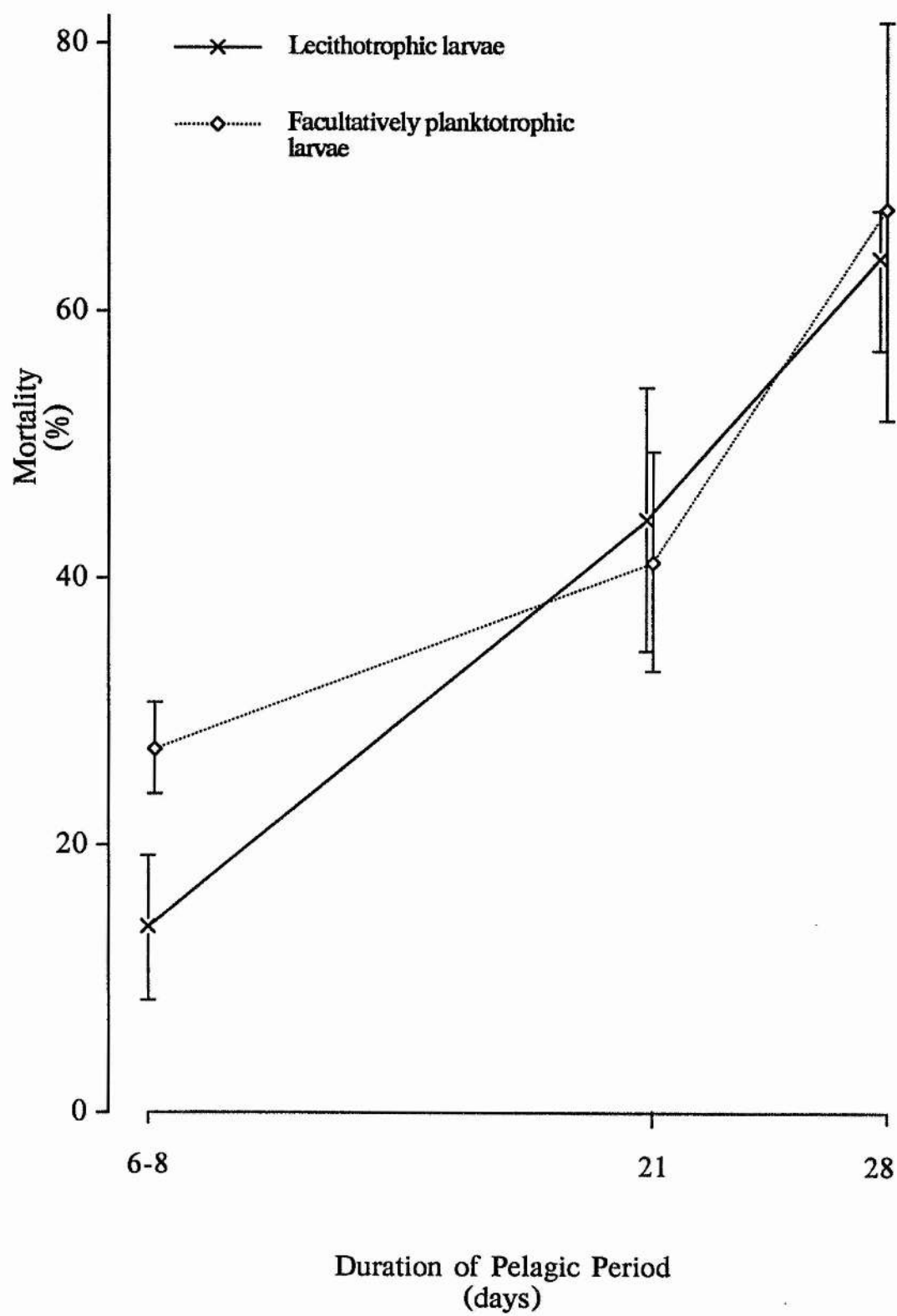
The mortality of those veligers which had spontaneously evacuated their larval shell was established to increase significantly with duration of the delay period ( $P < 0.001$ , see Table 5.3), yet remain unaffected by larval nutritional state ( $P = 0.231$ , see Table 5.3). This increase in mortality was significant both for lecithotrophic larval



**Table 5.3. Factorial Analysis of the Effects both of Duration of Delay Phase and Nutritional State (facultatively planktotrophic or lecithotrophic) on the Mortality of *Adalaria proxima*.** Presented are the results of two-way ANOVAs performed on veliger, spontaneous evacuee, metamorph and total cumulative mortality using a two-factor crossed GLM (general linear model). Asterisks denote those responses considered significant at the  $P < 0.05$  level. Duration of the delay phase has a significant effect only on the total mortality ( $P < 0.001$ ) and the mortality of spontaneous evacuees ( $P < 0.001$ ). Larval nutritional state has no effect upon any mortality parameter examined, and, in addition, the lack of any significant interactive term also indicates that nutritional state has no influence on the effect of delay phase on mortality.

	df	SS	MS	F-value	P
<i>Total Mortality</i>					
Duration of pelagic phase	2	6690.4	2585.8	12.96	<0.001*
Nutritional State	1	121.5	121.5	0.61	0.440
Interaction	2	242.1	121.1	0.61	0.550
Error	42	8382.8			
<i>Spontaneous Evacuee Mortality</i>					
Duration of pelagic phase	2	2557.9	1159.0	10.85	<0.001*
Nutritional State	1	157.9	157.9	1.48	0.231
Interaction	2	169.0	84.5	0.79	0.460
Error	42	4487.3	106.8		
<i>Metamorph Mortality</i>					
Duration of pelagic phase	2	1188.0	411.5	2.43	0.100
Nutritional State	1	461.9	461.9	2.73	0.106
Interaction	2	77.5	38.7	0.23	0.796
Error	42	7097.0	169.0		
<i>Veliger Mortality</i>					
Duration of pelagic phase	2	382.7	129.0	0.90	0.416
Nutritional State	1	87.0	87.0	0.60	0.441
Interaction	2	236.2	118.1	0.82	0.447
Error	42	6041.7	143.8		

**Figure 5.2. The Effect of Extended Pelagic Period on the Mortality both of Facultatively Planktotrophic and Lecithotrophic Larvae.** Data are back-transformed means (expressed in percentage terms) obtained from subsamples of eleven larval cultures (lecithotrophic larvae) and five cultures (facultatively planktotrophic larvae). Subsampled individuals were scored according to the criteria previously presented in Section 3.1 as live veligers, spontaneous evacuees and metamorphs, or dead veligers, spontaneous evacuees and metamorphs. Total mortality for each nutritional state was therefore composed of all dead veligers, spontaneous evacuees and metamorphs. The corresponding absolute values of mortality are displayed in Table 5.4. Whilst total mortality was established to increase significantly ( $P < 0.05$ ) with increasing pelagic period (see Table 5.3), this effect was considered to be significant only for lecithotrophic larval cultures (see Table 5.4).



**Table 5.4a. Subsample Mortality both of Fed (Facultatively Planktotrophic) and Unfed (Lecithotrophic) *A. proxima* larvae.**

Mortality was determined for each of the subsamples in which metamorphic success was quantified. Data are the back-transformed angular means (expressed in percentage terms) obtained from five facultatively planktotrophic and eleven lecithotrophic larval cultures. Integers in parentheses represent the back-transformed standard errors of the means. The constituent components of total mortality (expressed at percentages of the whole subsample) for both fed and unfed larval cultures are also displayed. Larvae were scored as dead veligers, spontaneous evacuees or metamorphs using the criterion previously presented in Section 3.1. Significant differences in mortality between delay phases (at the  $P < 0.05$  level) were established by one-way ANOVAs and are denoted by an asterisk. Subsequent multiple comparisons by Tukey's HSD test were performed on the arc-sine transformed data in order to identify these significant differences. The resulting Tukey Groupings are expressed within the table as superscript letters adjacent to the relevant mortality levels and denote those metamorphic responses considered significantly different at the  $P < 0.05$  level.

	Mortality (%)			
	4-8 days	21 days	28 days	ANOVA
<i>Lecithotrophic Larvae</i>				
Total Mortality	13.9 <sup>a</sup> (8.4-19.2)	44.4 <sup>b</sup> (34.6-54.5)	64.0 <sup>b</sup> (57.1-67.6)	P<0.001*
Veligers	4.8 (2.9-7.1)	7.8 (5.5-11.6)	13.9 (9.5-19.1)	P= 0.201
Spontaneous Evacuees	3.8 <sup>a</sup> (2.1-6.1)	10.2 <sup>a/b</sup> (6.5-14.5)	23.1 <sup>b</sup> (20.5-25.7)	P=0.001*
Metamorphs	4.9 <sup>a</sup> (3.7-7.1)	16.0 <sup>a/b</sup> (10.8-22.0)	19.6 <sup>b</sup> (14.5-25.3)	P=0.035*
<i>Facultatively Planktotrophic Larvae</i>				
Total Mortality	27.2 (24.3-31.1)	41.2 (33.1-49.5)	67.7 (51.9-81.6)	P= 0.052
Veligers	14.4 (8.2-22.0)	7.1 (3.3-12.2)	13.7 (7.5-21.3)	P= 0.637
Spontaneous Evacuees	4.1 <sup>a</sup> (2.3-6.4)	22.3 <sup>a/b</sup> (16-8-28.4)	25.8 <sup>b</sup> (16.2-36.7)	P= 0.037*
Metamorphs	2.9 (0.9-6.0)	7.7 (5.8-9.9)	8.4 (1.3-20.7)	P= 0.723

**Table 5.4b. ANOVA Tables for Each Constituent of Subsample Mortality both of Fed (Facultatively Planktotrophic) and Unfed (Lecithotrophic) *A. proxima* larvae.** Significant differences in mortality between delay phases (at the  $P < 0.05$  level) were established by one-way ANOVAs and are denoted by an asterisk. The absolute values for each constituent of mortality are presented in Table 5.4a..



	df	SS	MS	F- value	P
<i>Lecithotrophic Larvae</i>					
Total Mortality					
among delay periods	2	5491	2746	13.46	<0.001*
within delay periods	30	6119	204		
Veligers					
among delay periods	2	476	238	1.70	0.201
within delay periods	30	4208	140		
Spontaneous Evacuees					
among delay periods	2	1683	842	8.24	0.001*
within delay periods	30	3065	102		
Metamorphs					
among delay periods	2	1119	560	3.76	P=0.035*
within delay periods	30	4471	149		
<i>Facultatively Planktotrophic Larvae</i>					
Total Mortality					
among delay periods	2	1441	720	3.82	0.052
within delay periods	12	2265	189		
Veligers					
among delay periods	2	143	72	0.47	0.637
within delay periods	12	1834	153		
Spontaneous Evacuees					
among delay periods	2	1044	522	4.40	0.037*
within delay periods	12	1422	119		
Metamorphs					
among delay periods	2	146	73	0.33	0.723
within delay periods	12	2626	219		

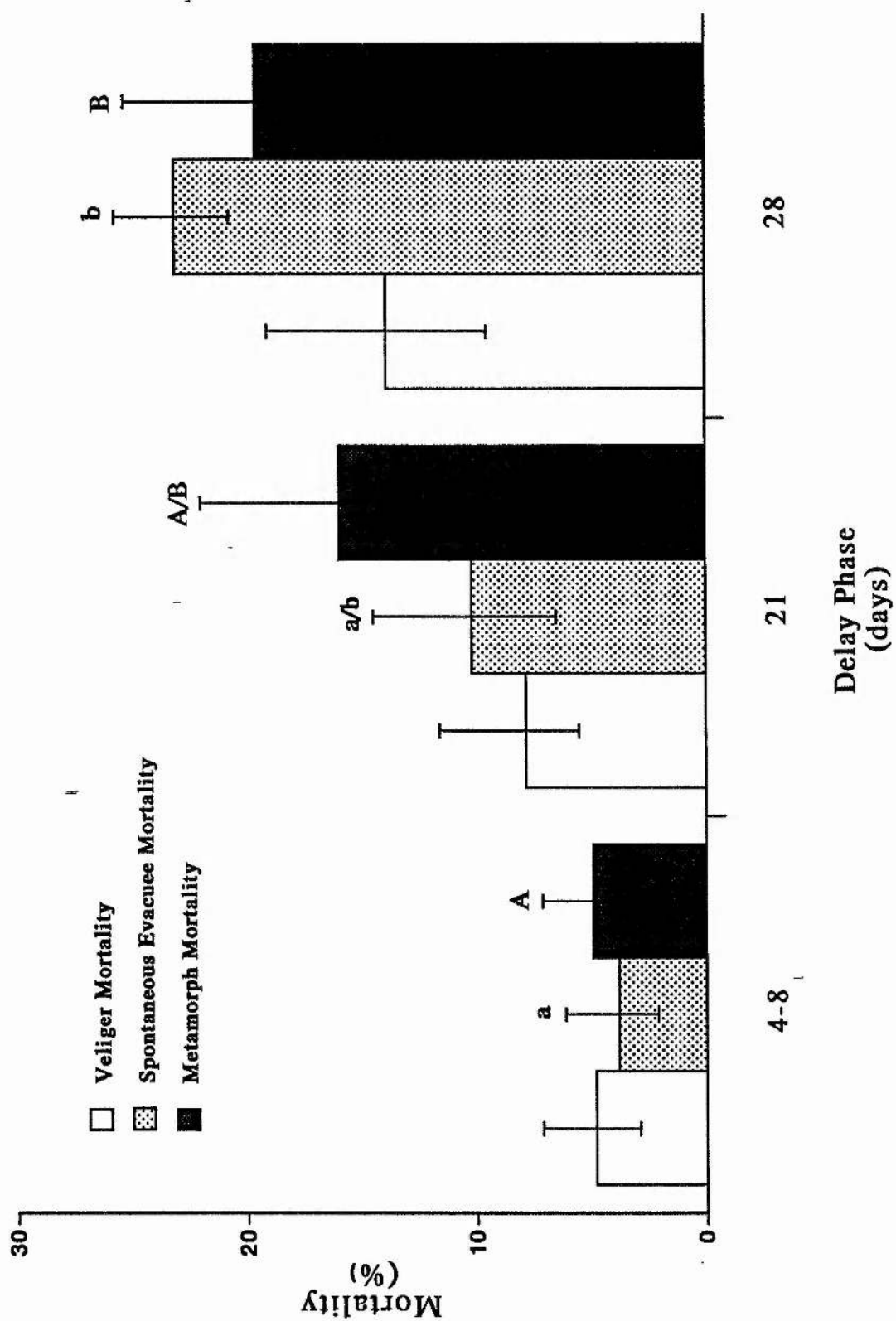
culture ( $P < 0.01$ , see Tables 5.4b) and facultatively planktotrophic larval cultures ( $P < 0.05$ , see Tables 5.4b). *A posteriori* multiple comparisons of spontaneous evacuee mortality among unfed larval cultures revealed the mortality level of 23.1% after a 28 day pelagic period to be significantly higher than the 3.8% mortality evident after 4-8 days delay, whilst the 10.2% mortality evident after 21 days was judged to be intermediate at the  $P < 0.05$  level (see Table 5.4a and Figure 5.3). The mortality of spontaneous evacuees from facultatively planktotrophic larval cultures is illustrated in Figure 5.4a and appears to follow the same trend, increasing from 4.1% after a 4-8 day delay up to a significantly higher 25.8% after a 28 day delay, again with an intermediate level of mortality after 21 days (see also Table 5.4b). Whilst for each delay phase the mortality of spontaneous evacuees from lecithotrophic larval cultures initially appears consistently less than for subsamples taken from facultatively planktotrophic cultures (see Tables 5.4a), larval nutritional state was, on examination, determined to exert no significant effect on the mortality of spontaneous evacuees subjected to an extended pelagic period ( $P = 0.460$ , see Table 5.3).

The proportion of unsuccessful metamorphs, that is, those found to be dead on inspection two days after exposure to an appropriate metamorphic stimulus ( $5 \cdot 10^{-3} \text{M}$  choline chloride), changed neither in response to the duration of the pelagic phase ( $P = 0.100$ ), nor in relation to larval nutritional status ( $P = 0.106$ , see Table 5.3). Separate one-way ANOVAs (see Table 5.4b) on the effect of delay phase on metamorph mortality did, however, reveal that although there was no significant increase in mortality evident among metamorphs from facultatively planktotrophic cultures ( $P = 0.723$ ), there was a marginally significant increase in the proportion of unsuccessful metamorphs originating from lecithotrophic larvae, from 4.9% after 4-8 days pelagic period up to 19.6% after 28 days ( $P = 0.035$ ). The Tukey groupings resulting from subsequent multiple comparison are illustrated both in Figure 5.3 and Table 5.3 and show a similar trend to that exhibited by spontaneous evacuees' mortality; namely that the extremes in delay phase also represent the extremes in mortality, and that the level of mortality evident after 21 days is intermediate between the two.

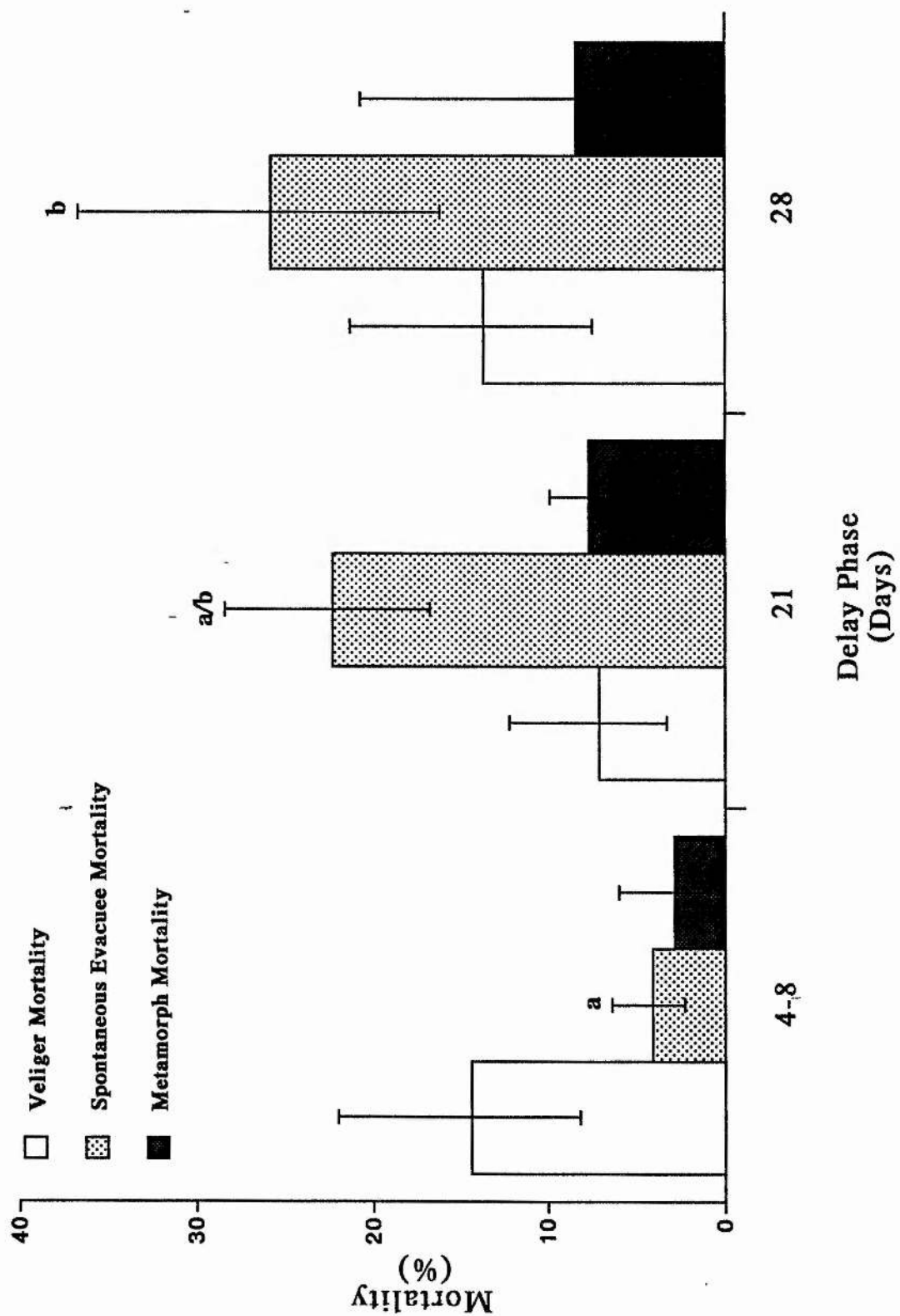
#### The Relationship Between the Mantle Length and Width of Newly Metamorphosed Juveniles

The relationship between the mantle length and mantle width of newly metamorphosed individuals was examined in order to test the validity of using mantle length as a reliable and non-destructive indicator of juvenile size. The result of a simple

**Figure 5.3. The Constituent Components of Lecithotrophic Larval Mortality.** Subsampled individuals were scored as dead veligers, spontaneous evacuees, or metamorphs. Data are back-transformed means (expressed in percentage terms) obtained from subsamples of eleven larval cultures. Absolute values, and the results of relevant one-way ANOVAs, are displayed in Table 5.4.



**Figure 5.4. Components of Facultatively Planktotrophic Larval Mortality.** Subsampled individuals were scored as dead veligers, spontaneous evacuees, or metamorphs. Data are back-transformed means (expressed in percentage terms) obtained from subsamples of five larval cultures. Absolute values, and the results of relevant one-way ANOVAs, are displayed in Table 5.4.



linear regression by least squares showing the relationship between newly metamorphosed juvenile mantle length and width is presented graphically in Figure 5.5 with the appropriate regression equation. The adjusted coefficient of determination ( $r^2$ -value) obtained was 46.4%, indicating that 46.4% of observed variation in mantle width (designated the dependent variable) can be attributed to mantle length (designated the independent variable). The  $r$ -value of 0.681, when compared with a critical value of 0.321 ( $n=100$ ), indicates that the linear relationship between metamorph length and width may indeed be considered significant. Additional analysis of the relationship provided by a regression ANOVA table also indicated mantle length to be important in explaining the variation observed in mantle width ( $F_{1, 98}=86.81$ ,  $P<0.001$ ). The use of mantle length as a reliable non-destructive indicator of newly metamorphosed juvenile size was therefore considered valid.

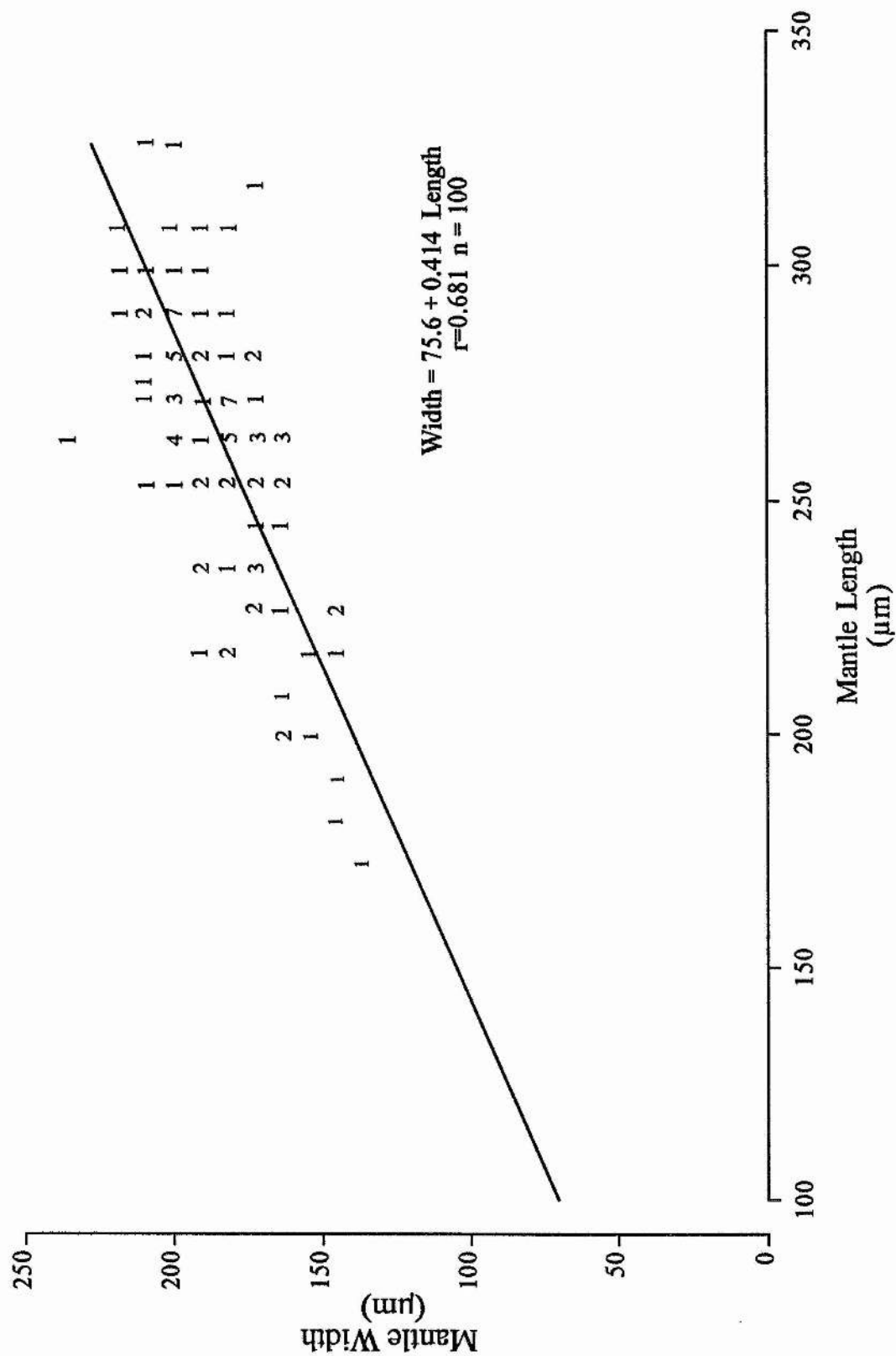
#### The Size of Newly Metamorphosed Juveniles

An extension of the pelagic phase resulted in a significant decrease in the mantle length of resultant newly metamorphosed juveniles ( $P<0.001$ , see Table 5.5a). In contrast, larval nutritional status (fed or unfed) had no significant effect on juvenile size ( $P=0.977$ , see Table 5.5a). The mantle lengths of metamorphs resulting from larvae raised within larval cultures supplied with food and subjected to a pelagic period of either 6 days, 21 days, or 28 days are displayed in Figure 5.6. The degree of between culture variation in mantle length apparent in Figure 5.6 was established to be nonsignificant ( $P=0.460$ ). For each of the five facultatively planktotrophic larval cultures a trend of decreasing mantle length with increasing delay phase is apparent. The mean mantle length of metamorphs resulting from facultatively planktotrophic larvae decreased significantly ( $P<0.01$ , see Table 5.5b) from 253 $\mu\text{m}$  after having undergone a pelagic phase of six days, to 190 $\mu\text{m}$  after a delay of 28 days (see Figure 5.8). A *posteriori* multiple comparison revealed that whilst mantle length decreased significantly between the six and 28 day delay periods, juveniles resulting from larvae subjected to a 21 day delay were neither significantly smaller than metamorphs resulting from a six day delay, nor significantly longer than the those subjected to a 28 day delay phase, but were considered to be of intermediate size (224.2 $\mu\text{m}$ , see Table 5.5b).

The mantle lengths of metamorphs resulting from larvae raised within unfed larval cultures and subjected to a pelagic period of either 6 days, 21 days, or 28 days are displayed in Figure 5.7. Once again, between experiment variation in mantle length was established to be nonsignificant ( $P=0.995$ ). A similar trend to that evident among facultatively planktotrophic larvae is apparent among metamorphs resulting from



**Figure 5.5 The Relationship between metamorph mantle length and mantle width.** Data were obtained for a total of 100 newly metamorphosed juveniles and analysed using simple linear regression by least squares. The P-value of  $<0.001$  ( $F_{1,98}=86.81$ ) resulting from ANOVA in addition to the adjusted  $r^2$ -value of 46.4% and corresponding correlation coefficient of 0.681 (compared to a critical value for  $n=100$  of 0.321) indicated mantle width to exhibit a significant relationship with mantle length.



**Table 5.5a. Factorial Analysis of the Effects both of Duration of Delay Phase and Larval Nutritional State (facultatively planktotrophic or lecithotrophic) on the Mantle Length of Resulting *A. proxima* Juveniles.** Mantle lengths were measured by ocular micrometer (see Plate 5.1) two days after administration of the artificial metamorphic inducer choline chloride ( $5 \cdot 10^{-3}M$ ). Presented are the results of two-way ANOVAs performed on mantle length data using a two-factor crossed GLM (general linear model) and subsequent one-way ANOVAs performed separately for the effects of delay phase on the mantle length of both lecithotrophic and facultatively planktotrophic larvae. Asterisks denote those responses considered significant at the  $P < 0.05$  level. It is apparent that duration of the delay phase has a significant effect on metamorph mantle length ( $P < 0.001$ ). Larval nutritional state has no effect upon the mantle length of the resulting juvenile ( $P = 0.977$ ). In addition, the lack of any significant interactive term ( $P = 0.241$ ) indicates that nutritional state has no influence on the effect of an increased pelagic period on metamorph size.

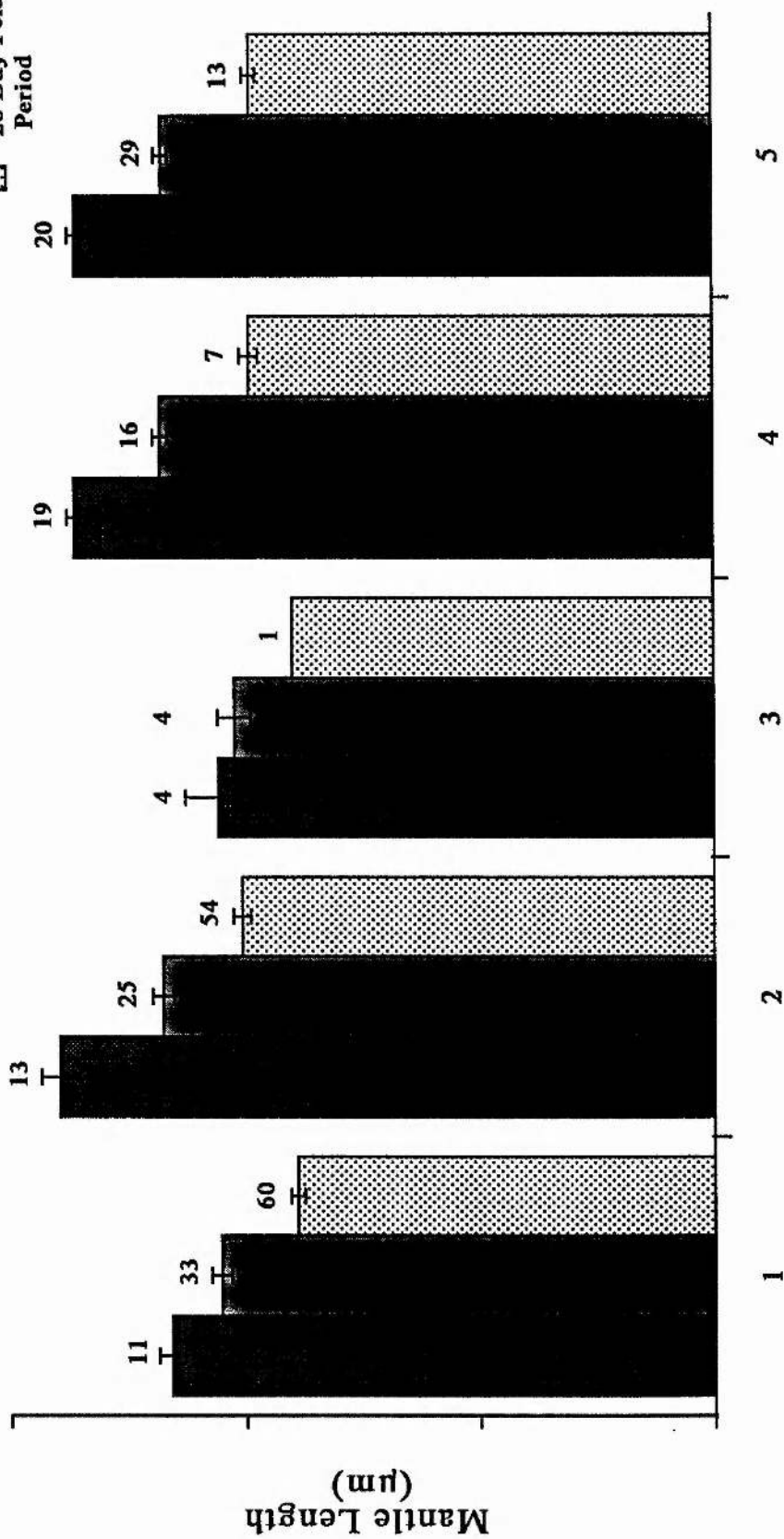
**Table 5.5b. The Mantle Lengths of Juvenile *A. proxima* Metamorphs Resulting both from Fed (Facultatively Planktotrophic) and Unfed (Lecithotrophic) larvae.** Data are mean values obtained from subsamples of five facultatively planktotrophic and eleven lecithotrophic larval cultures. The sizes of juveniles resulting from both facultatively planktotrophic and lecithotrophic modes of development decrease with increasing delay phase ( $P < 0.001$  and  $P < 0.01$  respectively). Subsequent multiple comparisons by Tukey's HSD test were performed in order to identify these significant differences. The resulting Tukey Groupings are expressed within the table as superscript letters adjacent to the relevant mortality levels and denote those metamorphic responses considered significantly different at the  $P < 0.05$  level.

	df	SS	MS	F-value	P
<i>Results of Two-way ANOVA by GLM</i>					
Duration of pelagic phase	2	37613.3	15110.6	28.81	<0.001*
Nutritional State	1	0.5	0.5	0.00	0.977
Interaction	2	1542.6	771.3	1.47	0.241
Error	42	22030.0	524.5		
<i>Results of separate One-way ANOVAs</i>					
Lecithotrophic larvae					
among delay periods	2	23534	1177	35.69	<0.001*
within delay periods	30	9891	330		
Facultatively planktotrophic larvae					
among delay periods	2	9723	4862	11.66	<0.01*
within delay periods	12	5004	417		

Nutritional Status of Larvae	Metamorph mantle length (μm)		
	4-8 days	21 days	28 days
Facultatively Planktotrophic	253.3 <sup>a</sup>	224.2 <sup>ab</sup>	190.9 <sup>b</sup>
Lecithotrophic	257.6 <sup>A</sup>	207.0 <sup>B</sup>	196.4 <sup>B</sup>

**Figure 5.6. The Effect of an Extended Pelagic Period on the Mantle Length of Metamorphs Resulting From Facultatively Planktotrophic Larval Cultures.** Mantle lengths were measured by ocular micrometer (see Plate 5.1) two days after administration of the artificial metamorphic inducer choline chloride ( $5 \cdot 10^{-3}M$ ). A general trend of decreasing mantle length with increasing delay phase is apparent. Data are mean values for each of the five facultatively planktotrophic (fed) larval cultures subjected to an enforced extended pelagic period. Integers above each column denote the number of metamorphs from which each mean was derived.

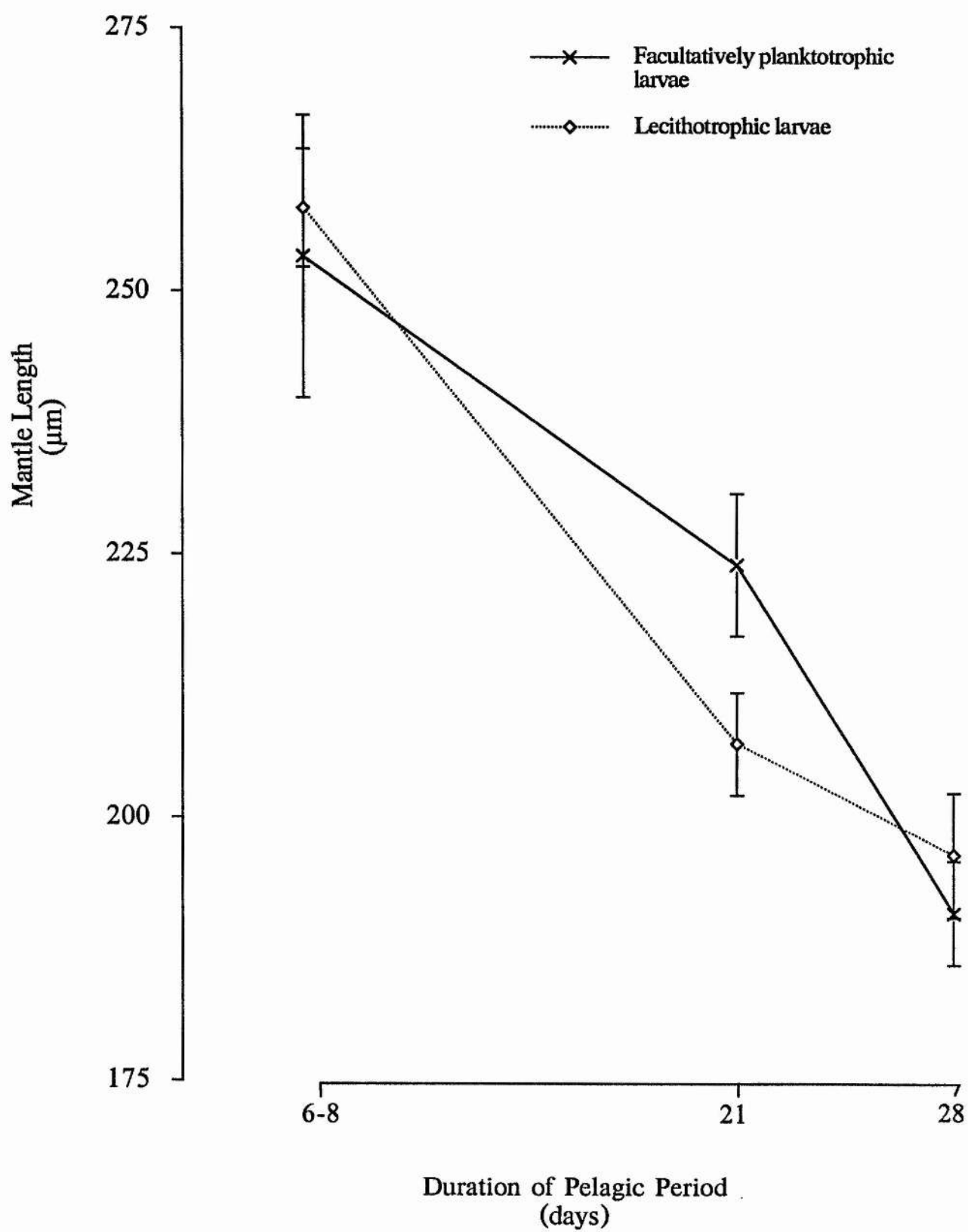
■ 6 Day Pelagic  
 Period  
 ■ 21 Day Pelagic  
 Period  
 ■ 28 Day Pelagic  
 Period



Larval Culture

**Figure 5.8. The Effect of an Extended Pelagic Period on the Mantle Length of Metamorphs Resulting From Lecithotrophic and Facultatively Planktotrophic Larval Cultures.** Data are mean mantle lengths obtained from subsamples of eleven larval cultures (lecithotrophic larvae) and five cultures (facultatively planktotrophic larvae). Mantle lengths were measured by ocular micrometer (see Plate 5.1) two days after administration of the artificial metamorphic inducer choline chloride ( $5 \cdot 10^{-3}M$ ). Analysis (see Table 5.5a.) of the effects both of delay phase and nutritional state on mantle length by two-way ANOVA (using GLM) established that whilst duration of the delay phase had a significant effect on mantle length ( $P < 0.001$ ), larval nutritional state did not ( $P = 0.977$ ). Neither did nutritional state have a significant influence on the effect of an increased pelagic period on metamorph size (interactive term  $P = 0.241$ ). Separate one-way ANOVAs and *a posteriori* multiple comparisons by Tukey's HSD (presented in Table 5.5b.) showed whilst a significant reduction in mantle length was apparent in metamorphs originating from both facultatively planktotrophic and lecithotrophic larval cultures after a delay phase of 28 d, only metamorphs resulting from lecithotrophic (unfed) larval cultures showed a significant decrease in mantle length after 21 d.



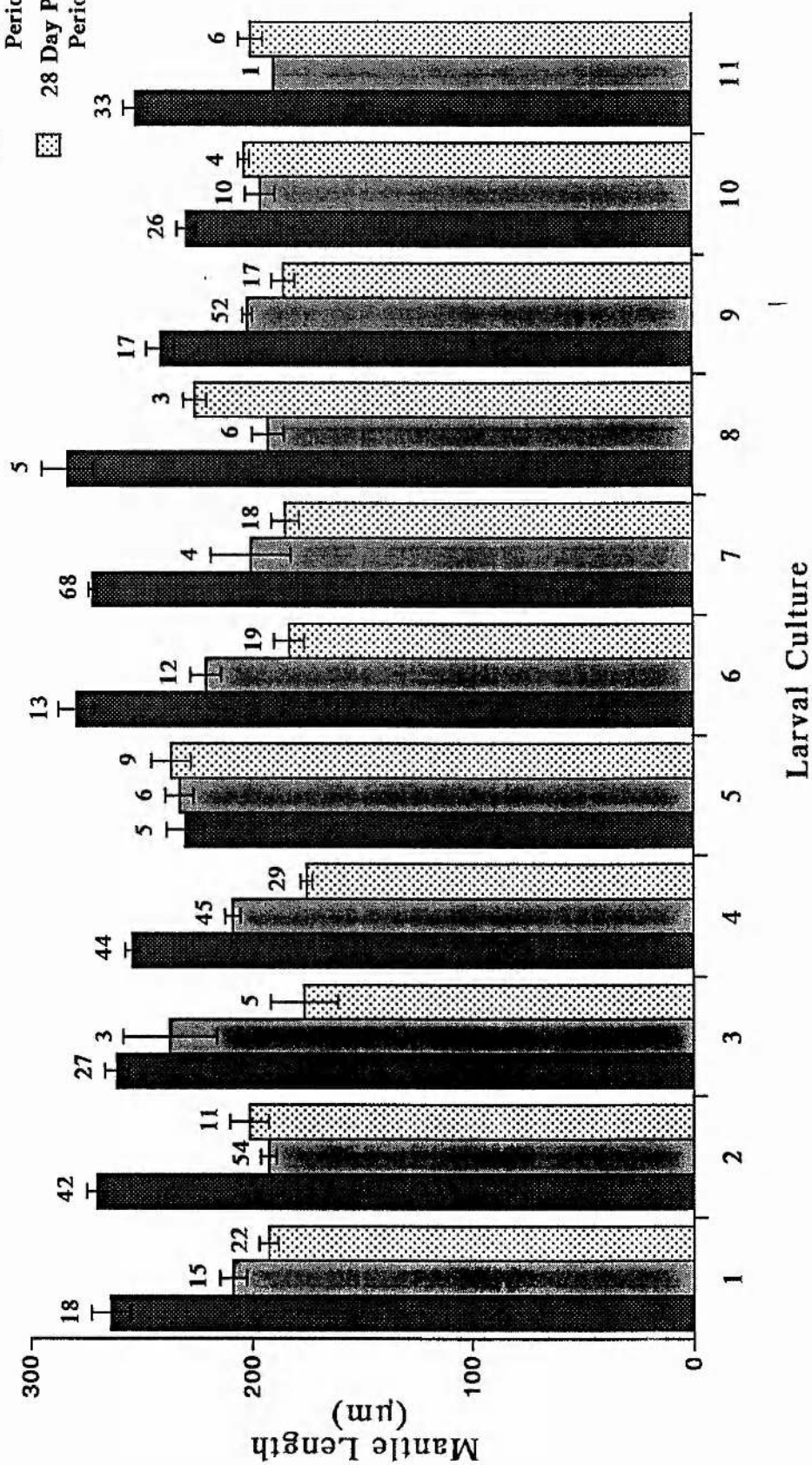


lecithotrophic larvae. With the exception of Culture 5 the size of juveniles from each culture tends to generally decrease with increasing delay phase (see Figure 5.7). Indeed, the mean mantle lengths of metamorphs resulting from lecithotrophic larvae decrease significantly with delay phase ( $P < 0.001$ , see Table 5.5a). In contrast to metamorphs resulting from facultatively planktotrophic larvae, the mantle length of juveniles resulting from lecithotrophic larvae decreases significantly only within the first 21 days of the delay phase (from 257.6  $\mu\text{m}$  to 207.6  $\mu\text{m}$ , see Table 5.5b). Whilst the mantle length continues to decrease thereafter to 196.4  $\mu\text{m}$  after a delay of 28 days, the decrease in mantle length resulting from this further drop is not considered significant (see Table 5.5b).

The similarity between the mantle lengths of juveniles resulting from facultatively planktotrophic and lecithotrophic larvae is illustrated by Figure 5.8. A two-way ANOVA examining the effects of delay phase and larval nutritional status confirmed this, showing nutritional status to have no significant influence on the effect of an increased pelagic period on metamorph size ( $P = 0.241$ , see Table 5.5a).

**Figure 5.7. The Effect of an Extended Pelagic Period on the Mantle Length of Metamorphs Resulting From Lecithotrophic Larval Cultures.** Mantle lengths were measured by ocular micrometer (see Plate 5.1) two days after administration of the artificial metamorphic inducer choline chloride ( $5 \cdot 10^{-3}M$ ). In a similar fashion to metamorphs from facultatively planktotrophic larval cultures (see Figure 5.6), there appears to be a general trend of decreasing mantle length with increasing delay phase (although see Larval Culture Five). Data are mean values for each of the eleven lecithotrophic (unfed) larval cultures subjected to an enforced extended pelagic period. Integers above each column denote the number of metamorphs from which each mean was derived.

6-8 Day Pelagic Period  
21 Day Pelagic Period  
28 Day Pelagic Period



## 5.4 DISCUSSION

### The Delay Capacity of *A. proxima*

This study has established that both fed and starved larvae of the nudibranch *Adalaria proxima* may successfully undergo an enforced pelagic phase of up to 28 days. Indeed, during the course of this study, a few individuals were observed to successfully metamorphose after a 31 day delay phase. Thompson (1958) first reported that larvae of *Adalaria proxima* could extend the 'searching phase', or pelagic period, for up to two weeks in the absence of an appropriate metamorphic stimulus. In a later study Todd & Havenhand (1985) found that starved *A. proxima* larvae could delay metamorphosis for as long as 10 days. Observations of larval longevity over a 14 day period led Kempf & Todd (1989) to estimate from mortality data the maximum delay phase for both fed and unfed larvae to be between 15 - 20 days. The present study has therefore extended the estimate of the maximum delay capacity of *A. proxima* by a further 8 - 11 days conferring a possible pelagic period of up to at least 28 - 31 days (at 10°C).

### Metamorphic Success

The artificial metamorphic induction agent choline chloride ( $5 \cdot 10^{-3}M$ ) was used to determine whether larvae possessed sufficient reserves to be capable of completing metamorphosis given the appropriate stimulus. This was considered a reliable functional indication of comparative larval physiological state after a known delay period. Successful metamorphs were considered to be those alive two days after administration of the metamorphic cue. The artificial cue choline chloride was employed in preference to the natural metamorphic cue because of the higher relative consistency in response observed during previous trials (see Chapters 3 & 4).

A trend of decreasing metamorphic success with increasing delay phase was apparent with the proportion of successful metamorphs resulting from lecithotrophic larvae falling 38.9% and facultatively planktotrophic 35.4% over the 28 day extended pelagic period.

The sole previous report of metamorphic success after a known pelagic period in *A. proxima* is an unreplicated and unrepeated observation provided by Kempf & Todd (1989). After 14 days  $^{12/35}$  (34.3%) of fed larvae successfully metamorphosed in response to the natural cue (*Electra pilosa* epiphytic on *Fucus serratus*) compared to  $^8/40$  (20.0%) of unfed larvae. Absolute comparisons with the present study are invalid due both to the qualitative nature of the observation and the use of the natural metamorphic inducer with its associated variability in response (see Chapter 4). It is, however, worthy of note in intimating a disparity in the level of metamorphic success between fed and starved larvae not evident within the present study. This higher metamorphic success displayed by the facultatively planktotrophic (fed) larvae led Kempf & Todd (1989) to propose that feeding may confer enhanced metamorphic success after an extension in the delay phase. Within the present study however, the proportion of successful metamorphs originating from facultatively planktotrophic larval cultures dropped from an initial 63.6% down to 51.9% after 21 days and to just 28.2% after a 28 day delay. In a similar fashion the metamorphic success of lecithotrophic larval cultures fell significantly in response to an extended pelagic period, from an initial 74.3% down to 49.9% after 21 days and just 35.4% after the maximum 28 day delay period ( $P < 0.001$ ). The hypothesis arising from the unrepeated observation of Kempf & Todd (1989) has not therefore been confirmed by the findings of the present study.

A decrease in metamorphic success in response to an extended pelagic period also has been observed in the tropical nudibranch *Phestilla sibogae* (Kempf & Hadfield, 1985). Perhaps the most comprehensive and comparable study concerning the effects of an extended larval period on the post-larval life history of a species with facultatively planktotrophic larvae is provided by Miller (1993). He found that an artificial extension in the delay phase of the nudibranch *P. sibogae* resulted in a host of negative effects on selected life-history parameters, including metamorphic success. A 28 day delay in metamorphosis resulted in a 75% decrease in the metamorphic success of lecithotrophic larvae compared to a 13% decrease in facultatively planktotrophic larvae. Whilst absolute comparisons of this tropical aeolid nudibranch with a mean generation time of 38 d (Harris, 1975), and *A. proxima*, an annual dorid nudibranch with a boreo-Arctic distribution are obviously invalid, a comparison of qualitative trends does prove instructive. A significant disparity between the metamorphic success of fed and starved *P. sibogae* larvae was established by Miller (1993). Larval nutritional status appeared to exert no such effect upon metamorphic success of *A. proxima* larvae within the present



study, although, in a similar fashion to that shown by *P. sibogae*, the metamorphic success of *A. proxima* larvae did decrease with extended pelagic period. A decrease in metamorphic success with increasing delay phase is not confined to molluscan larvae and has been documented also to occur among the Crustacea (Lucas et al., 1979) and Bryozoa (Woollacott et al. 1989). A reduction in metamorphic success does not necessarily constitute an inevitable result of an extension in delay phase however: larvae of the gastropod *Aplysia juliana*, for example, showed no decrease in metamorphic success with increasing pelagic period when exposed to the natural cue (Switzer-Dunlap, pers. comm., cited in Kempf, 1981)

Lucas et al. (1979) proposed that the observed decrease in metamorphic success of larval *Semibalanus balanoides* with an extension in delay phase could be attributed to the depletion of energetic reserves below a critical threshold level required for metamorphosis. The disparity between the metamorphic success of fed and starved larvae of facultatively planktotrophic species such as *Phestilla sibogae* (Kempf & Hadfield, 1985; Miller, 1993) would concur with this rationale. An additional mechanism capable of causing decreased metamorphic success with an extension of the delay phase was supplied by Miller (1993). He proposed that a proportion of unsuccessful metamorphs may possess incomplete or 'faulty' components in their metamorphic pathways and that an increase in such cases over time may be the result of tissue damage during the pelagic period which larvae lack the ability to repair. Indeed, many species of invertebrate larvae have been documented to exhibit changed morphology indicative of such tissue damage during an extended pelagic period - e.g. bivalves (Bayne, 1965), sea-urchins (Bonar, 1978) and sand-dollars (Highsmith & Emlet, 1986). If both depletion of energetic reserves and tissue damage contribute to unsuccessful metamorphosis, then the comparative metamorphic success demonstrated by fed and starved larvae of facultatively planktotrophic species may provide an indication of the relative importance of each factor. If both fed and starved larvae are subject to comparable levels of tissue damage, then the difference in metamorphic success between the two may be an indication of the effect of nutritional state on metamorphic failure, whilst the metamorphic success shown by fed larvae may be an indication of the importance of tissue damage on metamorphic failure. The significant difference in metamorphic success with nutritional state over time documented in *P. sibogae* would infer metamorphic success to be dominated by energetic requirements rather than tissue damage. Conversely, the absence of a significant difference in metamorphic success observed within the present study between fed and starved



larvae of *A. proxima* could be interpreted to indicate the relative importance of tissue degradation resulting in faulty metamorphic pathways as a contributing factor to the observed decrease in metamorphic success in this species.

#### The Effect of Larval Nutritional Mode on Survivorship

Larval feeding has been documented to extend the capacity to delay metamorphosis in several species of gastropod (Perron, 1981; Kempf & Hadfield, 1985; Miller, 1993). For example, fed larvae of the facultative planktotroph *Conus pennaceus* were shown by Perron (1981) to live longer in culture than unfed larvae. Several studies have examined the influence of feeding on the capacity of facultatively planktotrophic larvae to extend the competent phase. The effect of food supply on the ability to delay metamorphosis is well illustrated by the facultatively planktotrophic larvae of the opisthobranch *Phestilla sibogae* (Hadfield, 1972). Kempf & Hadfield (1985) showed that feeding could, in extreme cases, extend the competent period of *P. sibogae* by up to 90%. Starved larvae were shown to successfully metamorphose after a maximum delay phase of 22d, whilst fed larvae could withstand up to 42d of extended competent period. This was supported by Miller (1993) who found that whilst an enforced delay phase of 14d or more significantly decreased survivorship in both fed and starved larvae of *P. sibogae*, fed larvae displayed a higher survival rate than starved. Thus, larvae ingesting particulate food possessed a greater capacity to undergo an extended competent phase than those relying solely on endogenous energetic reserves.

An increase in the ability to delay metamorphosis by larval feeding does not appear to be universal among facultative planktotrophs. Both fed and starved larvae of the echinoid *Clypeaster rosaceus* displayed similar development times, although feeding did increase juvenile size, growth rate, and survivorship (Emlet, 1986b). This led the author to conclude that for this facultatively planktotrophic species at least, nutritional status has no effect on the capacity to delay metamorphosis.

Thompson (1958) first observed that whilst *Adalaria proxima* veliger larvae were capable of the ingestion of phytoplankters (*Chlorella stigmatophora* and *Isochrysis galbana*) into the lumen of the left diverticulum, feeding during the pelagic phase was not a prerequisite for successful metamorphosis. Thompson was therefore the first to denote *A. proxima* as a facultative planktotroph even before

the term was coined, although he did not document whether the capacity of larvae to delay metamorphosis was affected by feeding.

Todd & Havenhand (1985) subjected unfed *Adalaria proxima* larvae to a delay in metamorphosis and postulated that larval feeding could be expected to increase the capacity to delay metamorphosis. The first comparative study of the delay capacity of both fed and starved *A. proxima* veliger larvae was performed by Kempf & Todd (1989). Larvae raised as facultative planktotrophs were fed with a mix of the phytoplankton species *Pavlova lutheri*, *Isochrysis galbana* and *Rhodomonasp*. Unexpectedly, Kempf & Todd (1989) found fed larvae to be less likely to survive an extension in delay phase than unfed veligers, although this difference in delay capacity was not judged to be significant. The decreased survivorship of fed *A. proxima* veligers was attributed to an artefact of the laboratory larval culture process. Larvae were held within the confines of petri dishes, and the addition of the algal food source to this restricted volume of water resulted in bacterial contamination and clogging of larvae by detrital matter. The authors concluded that whilst larval feeding could supplement endogenous energetic reserves, it did not enhance larval longevity.

A clear trend of increasing total mortality with increasing delay phase was evident for both larval developmental modes within the present study. The increase in total mortality with delay phase was considered to be significant for only lecithotrophic larvae ( $P < 0.001$ ) however, whilst for facultatively planktotrophic larvae the increase in mortality was statistically marginal ( $P = 0.052$ ). Further examination revealed that whether *Adalaria proxima* larvae were cultured as facultative planktotrophs (fed) or as lecithotrophs (unfed) had no significant effect on any of the subsequent mortality parameters assessed (veliger, spontaneous evacuee, metamorph or total mortality). Furthermore, and again in contrast to the findings of Miller (1993) on *Phestilla sibogae*, the nutritional state of *A. proxima* larvae appeared to have no influence on the severity of effect caused by an extended delay phase.

The absence of any established enhancement in ability to delay metamorphosis with larval feeding by *Adalaria proxima* would not appear to be due an inability to digest and assimilate the larval food source. This is evidenced by the comparison of the physiological state of fed and starved larvae provided by Kempf & Todd (1989). Using structural and biochemical data they established *A. proxima* to be a true facultative planktotroph, capable of, although not dependent upon, the

supplementation of endogenous energetic (yolk) reserves through the assimilation of exogenous particulate matter. That veliger larvae of *A. proxima* possess a digestive system structurally similar to that of obligate planktotrophic species, and would therefore be anatomically capable of feeding, was established by ultrastructural examination. Obligate planktotrophic larvae possess visible heterophagosomes within the left digestive diverticulum, and in addition to a well developed oesophagus, stomach, left digestive diverticulum and intestine, Kempf & Todd (1989) also established the presence of heterophagosomes within the left digestive diverticulum of *A. proxima*. That *A. proxima* larvae are capable of actually assimilating an exogenous particulate food source was demonstrated by the disparate biochemical composition of larvae cultured as lecithotrophs (unfed) and facultative planktotrophs (fed). Quantitative comparisons of the major biochemical constituents - protein, carbohydrate and lipid - were made for veliger larvae having undergone a 10 day pelagic period. Whilst protein content remained unchanged over the ten day period, both the lipid and carbohydrate contents of larvae were shown to decrease, with lecithotrophic larvae displaying a greater loss than that shown by facultatively planktotrophic. Summation of the constituent biochemical fractions and conversion to joule equivalents revealed that whilst larvae from both nutritional treatments lost energy over the ten day extension of the pelagic phase, starved larvae appeared to lose more than fed. The relative energetic losses demonstrated by larvae during the pelagic phase led Kempf & Todd (1989) to conclude that whilst veliger larvae of *Adalaria proxima* could indeed successfully assimilate particulate matter from the environment, feeding could not fully compensate for the metabolic requirements of pelagic larvae. Starved larvae were demonstrated to lose 25% of their mean original energetic content (at hatching) over the ten day enforced pelagic period (falling from  $16.4 \cdot 10^{-3} \text{ J} \cdot \text{larva}^{-1}$  at hatching down to  $12.3 \cdot 10^{-3} \text{ J} \cdot \text{larva}^{-1}$ ), whilst fed larvae exhibited a loss of just 16% (down to  $13.7 \cdot 10^{-3} \text{ J} \cdot \text{larva}^{-1}$ ). Feeding therefore reduces the  $4.1 \text{ J} \cdot \text{larva}^{-1}$  of endogenous energetic reserves lost over the course of the experiment to  $2.7 \cdot 10^{-3} \text{ J} \cdot \text{larva}^{-1}$ . Thus, assimilation of exogenous matter during the 10 day pelagic period in *A. proxima* would, using the figures supplied by Kempf & Todd (1989) appear to compensate for only 34.2% ( $1.4 \cdot 10^{-3} \text{ J} \cdot \text{larva}^{-1}$ ) of the total larval energetic requirement. This is in contrast to the findings of Kempf & Hadfield (1985) who found that the facultatively planktotrophic larvae of the tropical nudibranch *Phestilla sibogae* could successfully meet all larval energetic requirements by feeding upon phytoplankton.

The findings of Kempf & Todd (1989) demonstrate that whilst *Adalaria proxima* larvae may feed, energy will continue to be lost albeit at a lower rate than that in starved larvae. This would imply that the capacity of *A. proxima* to withstand a delay in metamorphosis in this species, although it may be theoretically extended by larval feeding, would not be indefinitely prolonged. The experimental results obtained by Kempf & Todd (1989) did not, however, support this assumption, and indeed showed a trend (although statistically nonsignificant) of decreased longevity among fed larvae. The present study also failed to establish a significant difference in the mortality and longevity exhibited by fed and starved larvae. Facultative planktotrophy has been proposed to be an evolutionary intermediate stage in the shift between obligate planktotrophic and lecithotrophic development (Strathmann, 1978; Kempf & Hadfield, 1985; Todd, 1987; Kempf & Todd, 1989). The apparent unimportance of larval feeding during the pelagic phase of *A. proxima*, notwithstanding the possession of a functional digestive system, may be indicative of the transitional evolutionary nature of facultative planktotrophy as a mode of development. The relatively ineffective nature of larval feeding in this species may in addition lend support to the hypothesis presented by Todd (1986) who proposed that the functional larval gut may not have been retained from the putative planktotrophic ancestral state to enable larval feeding, but rather, to enable the newly metamorphosed juvenile to graze upon detritus. Juvenile and adult *A. proxima* feed upon the bryozoan *Electra pilosa*, and the newly metamorphosed juvenile (mantle length 300µm) will be under a prey-size constraint, unable to penetrate the protective zooid frontal membrane with its radula. The provision of a digestive system adapted to a herbivorous diet may therefore allow the juvenile to graze upon the associated detritus/surface film for 1 - 3 weeks until a sufficient body size is attained for the nudibranch to successfully pierce the frontal membrane and consume the zooid contained within.

The study of facultatively planktotrophic larvae allows a comparison of the effect of an extended delay phase upon larvae of different development modes. The lack of exogenous energetic supplementation in lecithotrophic larvae could intuitively be expected to exacerbate any negative consequences of a delay in metamorphosis when compared to fed conspecifics. Whilst there did appear to be a general trend of lecithotrophic larvae suffering more extreme effects than fed larvae in the parameters tested within this present study, no such expected significant differences between larval nutritional states was established for any of the parameters examined. In the study of facultatively planktotrophy in *A. proxima*



performed by Kempf & Todd (1989) only 34% of the shortfall in larval metabolic demands during the delay phase was determined to be supplemented by feeding. Therefore *A. proxima* is accepted to be capable of facultative planktotrophy, but unlike *P. sibogae* it is not fully able to supplement energetic reserves with larval feeding (see Kempf & Todd, 1989). Whilst it is possible that the laboratory diet (a mix of three phytoplankton species) may have been sub-optimal, this is still a relatively minor amount. Such a restricted ability to supplement of endogenous energetic reserves might well account for the lack of difference observed between fed and starved larvae. The results of this present study, that larval nutritional status does not appear to confer significant advantages during the pelagic phase, confirm that *A. proxima* may be regarded as a restricted planktotroph. It may be that the expected advantage of possessing a functional larval gut is conferred in overcoming the prey-size constraint associated with the post-metamorphic juvenile phase (see Todd, 1986) rather than, as is more conventionally assumed, in the pelagic phase.

#### The Fate of Veligers Subjected to an Extended Pelagic Period

More information concerning the fate of veligers subjected to an extended pelagic period and subsequently exposed to an appropriate metamorphic inducer was obtained by considering the levels of mortality evident among the constituent components of total subsample mortality. Both metamorphic success (previously discussed) and mortality were determined for those larvae which survived in culture until subsampled. The status of larvae within cultures was monitored and mortality observed to increase over time, although no quantitative assessment of overall larval culture survivorship (i.e. proportion of hatched larvae remaining) was made. Cultures displaying mortality sufficiently high that no larvae remained up to the maximum delay period (28 d) were excluded from the analysis, and mortality among remaining cultures appeared comparable. Whilst an appreciation of the proportion of hatched larvae surviving in culture after a known delay may have been informative, the present study focused on what was considered a more functional parameter : the ability of surviving veligers to respond to a suitable metamorphic inducer.

The mortality of veligers subjected to  $5 \cdot 10^{-3} \text{M}$  choline chloride did not increase with duration of the delay phase, although total mortality did. This would imply veligers do not just exhaust their energetic reserves and die, but rather change

status and subsequently die as spontaneous evacuees or unsuccessful metamorphs. This is supported by the trend of increasing spontaneous evacuee and metamorph mortality observed among both fed and starved larvae. For both lecithotrophic and facultatively planktotrophic larvae there are greater absolute levels of, and a more significant increase in, the mortality of spontaneous evacuees relative to metamorphs after an extension of the delay phase. For example, for lecithotrophic larvae the proportion of unsuccessful metamorphs increased from an initial 4.9% up to 19.6% ( $P=0.035$ ) after a 28 day delay, whilst the proportion of dead spontaneous evacuees increased from 3.8% to 23.1% ( $P=0.001$ ). In a similar fashion, the proportion of unsuccessful metamorphs originating from facultatively planktotrophic larvae increased from an initial 2.9% to just 8.4% ( $P=0.732$ ) whilst the proportion of dead spontaneous evacuees increased from 4.1% to 25.8% ( $P=0.037$ ). These results would infer that on encountering a suitable cue after having undergone an extended pelagic period, a veliger possessing insufficient energetic reserves (whether purely endogenously derived or supplemented by particulate feeding) to successfully metamorphose will not remain as a veliger. Rather, the larva will be compelled to undergo distinct morphological changes. As the duration of the delay phase increases, however, it will become more likely that these morphological changes will not culminate in successful metamorphosis, and that the majority of larva either will die in a state resembling that of a spontaneous evacuee, whilst a minority will die upon (apparent) morphological completion of metamorphosis.

#### The Size of Newly Metamorphosed Juveniles

The size of newly metamorphosed juveniles as indicated by the maximum mantle length decreased significantly following an extension in the duration of the pelagic phase ( $P<0.001$ ). In a similar trend to that observed for all other parameters within this study, larval nutritional state was not established to have an effect on the size of resulting metamorphs. The mantle lengths of juveniles resulting from lecithotrophic and facultatively planktotrophic larvae decreased from initial means of  $258\mu\text{m}$  and  $253\mu\text{m}$  respectively down to minimum mean lengths of  $196\mu\text{m}$  and  $190\mu\text{m}$  after the maximum pelagic period of 28 days. It is, however, interesting to note that in metamorphs resulting from larvae raised as lecithotrophs, juvenile size decreased significantly within the first 21 days of delay; in larvae raised as

facultative planktotrophs, a significant decrease was not evident until metamorphosis had been delayed for the maximum pelagic period of 28 days. The faster decrease in juvenile size observed among metamorphs resulting from starved larvae may be a reflection of the absence of any supplementation of endogenous energetic reserves in the pelagic stage.

The settlement size of lecithotrophic larvae has been documented to decrease with increasing delay phase in several species (Kempf & Hadfield, 1985; Emlet, 1986; Highsmith & Emlet 1986; Richmond, 1987; Miller, 1993). The size of an individual at settling is of ecological importance because a larger size at metamorphosis has been proposed to confer an increase in the competitive ability of an individual (Spight, 1976; Rivest, 1983) and a reduction in the likelihood of death by micro-carnivore predation (Spight, 1976; Rivest, 1983; Werner, 1986; Hadfield & Miller, 1987; Rowe & Ludwig, 1991). An additional cost will be incurred if the individual cannot 'catch up' and therefore remains retarded in development and/or growth relative to conspecifics of the same age. Growth rate may not necessarily be compromised by an extended pelagic period, and has been demonstrated to be unaffected by an extension of the pelagic period in the gastropods *Crepidula fornicata* (Pechenik & Eyster, 1989) and *Aplysia juliana* (Kempf, 1981), the bivalve *Crassostrea virginica* (Newkirk et al., 1977), the echinoderms *Dendraster excentricus*, *Echinorachnius parma* (Highsmith & Emlet, 1986) and *Strongylocentrotus droebachiensis* (Rumrill, 1989). There are, however, sufficient examples of reduced post-metamorphic growth rates in response to extended pelagic periods (Neilson, 1981; Woollacott et al., 1989, see also Table 5.1), to indicate that juveniles having undergone an extended larval phase may never catch up with peers and thus will remain at a competitive and/or reproductive disadvantage.

The most taxonomically proximate organism to *A. proxima* for which relevant information is available is the nudibranch *Phestilla sibogae*, which reacted to an extension of the pelagic phase with a reduction in settlement size (Miller, 1993) in a manner similar to that observed in *A. proxima*. This decrease in settlement size was proposed by Miller (1993) to be the cause of the observed increase in juvenile period (time from metamorphosis to initiation of egg laying), evident in individuals having undergone an extended delay phase. Larger juveniles would attain the minimum required size for spawning before growth was completed, eventually resulting in a larger more fecund adult. Conversely, smaller



juveniles would attain this minimum size nearer to developmental completion, resulting in smaller and less fecund adults.

One of the original aims of the present study was to follow the growth rates of metamorphs resulting from larvae which had been subjected to a known extended pelagic period. Unfortunately each larval culture was subjected to repeated subsampling, and therefore, by necessity, subsample numbers were restricted. In addition, culture conditions/techniques were such that repeated attempts over the 1993 and 1994 spawning seasons were unsuccessful. Consequently, the effect of the reduction in juvenile size observed among *A. proxima* veligers subjected to a delay in metamorphosis on subsequent life history is unknown. However, the effect of an extended pelagic period on *A. proxima* (increased mortality, decreased metamorphic success, and reduction in juvenile size) appears strikingly similar to *P. sibogae*, and it may be that post-metamorphic growth rates follow a similar trend, intimating that small *A. proxima* juveniles remain at a competitive disadvantage.

#### The Ecological Significance of Delayed Metamorphosis in *Adalaria proxima*

The ecological significance of a capacity to delay metamorphosis until encountering the appropriate metamorphic stimulus/stimuli is dependent upon the fitness of those individuals which have undergone a delay in metamorphosis (Pechenik 1985). An extension of the pelagic phase may result in a reduction in individual fitness by increasing parameters such as larval and juvenile mortality and by causing reduced metamorphic success, decreased juvenile growth rate and reduced fecundity. If such negative effects on subsequent life history parameters occur, then although delayed metamorphosis may be demonstrated within the laboratory, its ecological significance will be restricted as individuals having undergone a delay in metamorphosis will make little or no genetic contribution to the next generation.

This study has established that although *Adalaria proxima* may delay metamorphosis for up to 31 d, an extension of the pelagic phase does indeed result in a host of negative effects such as increased larval mortality, decreased metamorphic success and a smaller size at metamorphosis. This would indicate that resulting juveniles will indeed show restricted competitive ability and a

compromized level of fitness, and that the laboratory determined ability to delay metamorphosis is of restricted or minor ecological relevance for *A. proxima*. Further evidence in support of this conclusion is offered by the genetic profiles of several Scottish *A. proxima* populations monitored over three generations by Todd *et al.* (1988). Gene flow, and therefore the genetic homogeneity of populations, is increased by larval dispersal (Scheltema, 1978; Crisp, 1974, 1978), and indeed, dispersal has been previously widely proposed as the primary role of the pelagic larva (Crisp, 1974, 1976b; Christiansen & Fenchel, 1979). Whilst *A. proxima* may delay metamorphosis for up to 31 d, significant genetic heterogeneity between populations as little as 3 km apart was determined using gel electrophoresis. Todd *et al.* (1988) estimated the dispersal potential of *A. proxima* to be in the order of  $10^1$  -  $10^2$  km, conferring genetic differentiation between populations on a scale of  $10^2$  -  $10^3$  km. Evidence of such small scale differentiation of populations therefore conflicted with the estimated dispersal capacity of *A. proxima* larvae based upon the maximum delay phase determined within the laboratory.

The restricted ecological significance of delayed metamorphosis in *A. proxima* indicated by the number of negative effects on subsequent life history parameters established in this study would help to explain the unexpectedly restricted genetic exchange observed in this species. Several theories, none of which may be mutually exclusive, were advanced by Todd *et al.* (1988) to explain the high degree of genetic diversity among, and by implication, low degree of gene flow between, tidally connected populations. The authors concluded perhaps the most-probable explanation to be that larval transport was restricted because *A. proxima* larvae do not exercise their potential to undergo a delay in metamorphosis and instead settle and metamorphose on, or soon after, the attainment of competence. This was further supported by a refined study of population genetics performed by Todd *et al.* (1994), which focused specifically upon small-scale genetic differentiation among populations of *A. proxima*. The authors found that populations sited on the west coast of Scotland and separated by as little as 90 m displayed significant genetic differentiation. This was interpreted as providing further support for the earlier conclusion of Todd *et al.* (1988); namely that larvae omit the pelagic period and therefore settle before being subjected to dispersal.

An additional explanation for the unexpectedly low degree of gene flow between geographically proximate locations may be supplied by the present study. *A. proxima* larvae may realise their potential for delayed metamorphosis in the field

but competitive ability and subsequent fitness may be compromised to such an extent that immigrants will have a nonsignificant effect on the genetic profile of the population.

## CHAPTER 6

### GENERAL DISCUSSION

#### The Ontogeny of Competence in *Adalaria proxima*

The attainment of competence denotes the completion of essential developmental changes required preparatory to the shift from pelagic larval to benthic juvenile modes of life (Miller & Hadfield, 1986) and as such may be considered a fundamental defining life history stage within marine invertebrates. By employing a range of metamorphic induction agents, both natural and artificial, this study has established that *Adalaria proxima* displays cue-specific competency. The first, and only other, example of cue specific competency within the Mollusca is provided by larvae the prosobranch *Crepidula fornicata*. Using both the natural cue (adult-conditioned sea water) and 20mM excess potassium, Pechenik and Gee (1993) found larvae attained competence to respond to elevated potassium ions 12-24h prior to attaining competence in response to the natural cue (Pechenik & Gee, 1993). Whilst this was interpreted as indicative of different sites of action for the natural cue and potassium, it may be that potassium acts at a later stage in the same pathway as the natural cue.

The findings of the present study are distinct from those of Pechenik & Gee (1993), however, in establishing that choline- and not potassium-mediated metamorphosis may be successfully induced at an ontogenetically earlier stage than the appropriate natural cue. This finding may be considered to be of significance for two reasons. First, the accurate diagnosis of competence within the laboratory is of importance both in the investigation of morphogenic cues and when determining the capacity of species to delay metamorphosis. Therefore the empirically determined attainment of competence within the laboratory must be defined in relation to the relevant cue. Second, the development of competence in response to choline, prior to both elevated external potassium and the natural cue, may provide information concerning the mechanism of choline-mediated metamorphosis and the ontogeny of the metamorphic pathway(s) in *Adalaria proxima*. The insensitivity of embryos to elevated potassium and the natural cue relative to the efficacious nature of choline may indicate the choline-mediated metamorphic pathway (or the portion of the natural metamorphic pathway triggered

by choline) to be functionally complete at an ontogenetically earlier stage than the formation of the larval chemoreceptors.

Much work has been performed upon the development of competence in marine invertebrates using artificial induction agents and a comprehensive body of data is now available (see reviews of Hadfield, 1978a; Pawlik, 1990, 1992; Rodriguez *et al.*, 1993). However, only a limited amount of knowledge concerning the development of the metamorphic pathway may be provided by such crude tools as artificial induction agents. Future knowledge of the developmental mechanisms involved in the onset of competency may be provided by studies such as that of Degnan & Morse (1995), which address the morphogenesis of the metamorphic pathway by examining developmental gene regulation.

#### The Natural Metamorphic Cue of *Adalaria Proxima*

Molluscan species which exhibit highly specialized adult prey requirements (stenophagy) are frequently found to settle and metamorphose in close association with their prey (Scheltema, 1974; Hadfield, 1984; Hadfield & Switzer-Dunlap, 1984; Hadfield & Pennington, 1990; Pawlik, 1992; Havenhand, 1991; Rodriguez *et al.*, 1993) and members of the Nudibranchia are no exception (Perron & Turner, 1977; Chia & Koss, 1978; Hadfield, 1978a; Rodriguez *et al.*, 1993). There are however, notable exceptions to the putative relationship between catholicity of diet and degree of metamorphic cue specificity (Birkeland *et al.*, 1971; Hubbard, 1988; Pawlik, 1989, 1992), whilst other possible examples may suffer by the absence of systematic testing of other possible morphogenic substances (Carroll & Kempf, 1990). *Adalaria proxima* exhibits a relatively stenophagous adult diet (Thompson, 1958a; Todd *et al.*, 1991; Lambert & Todd, 1994), preying primarily upon the cheilostome bryozoan *Electra pilosa*. The present study provides evidence that, in common with many other stenophagous species, *A. proxima* displays a high degree of cue specificity and may successfully metamorphose in the field only in response to the principal adult prey item.

Further investigation was designed to facilitate future isolation and identification of the natural metamorphic cue emanating from *Electra pilosa*. Evidence is provided that natural cue-mediated metamorphosis may be effected in a manner different to that of elevated potassium, requiring just 30 minutes exposure in contrast to continual exposure. Further, the induction of metamorphosis appears



to be dose dependent in *A. proxima*, the optimal response being elicited by undiluted or 50% strength conditioned sea water, and the minimum effective dose being 1% of original strength. Whilst the absolute concentrations obviously cannot be determined, the findings of the present study are considered pertinent in the development of reliable bioassay procedure.

The morphogenic properties of microbial films (see reviews of Meadows & Campbell, 1972a; Crisp, 1974; Scheltema, 1974; Bonar *et al.*, 1986; Pawlik, 1992 and Rodriguez *et al.*, 1993) have been extensively reported within the literature for a wide range of marine invertebrate taxa (Cameron & Hinegardner, 1974; Maki & Mitchell, 1985; Hofmann & Brand, 1987; Maki *et al.*, 1989; Johnson *et al.*, 1991; Pearce & Scheibling, 1991; Johnson & Sutton, 1994; Leitz *et al.*, 1994). However, no evidence for a bacterial rôle in the induction of larval metamorphosis in *A. proxima* was found, and the effective elimination of morphogenic activity by 0.22µm filtering may suggest the cue molecule to be associated with larger organic complexes within the water column. Whilst investigations focusing upon natural metamorphic inducers are increasingly being documented in the literature, information is largely limited to source and effect. The identification and isolation of such morphogenic substances has previously been identified as a research priority (Hadfield, 1986; Chia, 1989; Pawlik, 1990, 1992; Rodriguez *et al.*, 1993), yet identification of natural cue substances remains rare - indeed, the first isolation and identification of a nudibranch metamorphic cue remains to be made. The way forward may now be provided by combination of the skills of larval ecologists and the analytical techniques of chemical ecologists.

#### The Effective Dispersal Potential Of *Adalaria Proxima*

The pelagic period possessed by the larvae of sessile or sedentary marine invertebrates constitutes a defining parameter in the population ecology of that species. For example, dispersal potential has been correlated with the duration of the larval pelagic period, and will consequently be greater in species which possess pelagic larvae relative to species which brood their young (Jablonski, 1986; Jackson, 1986; Scheltema, 1986; but see also Hedgecock, 1986). Employing this rationale previous authors have consequently attributed dispersal potential to influence such fundamental parameters as the colonization potential, and hence,

geographic range, and evolutionary longevity of a species (Scheltema, 1971, 1977, 1978; Jablonski & Lutz, 1983).

*Adalaria proxima* produces pelagic facultatively planktotrophic larvae which display a precompetent phase of 24 - 48h (Thompson, 1958a), although considerable variation in response to the natural cue has been observed (Todd *et al.*, 1988; Lambert & Todd, 1994). The observation that metamorphosis may be delayed for up to 14d (Kempf & Todd, 1989) led Todd *et al.* (1994) to estimate the potential dispersal capacity of this species to be in the order of  $10^1$  -  $10^2$  km (Todd *et al.* 1988), and the maximum extended pelagic period of 31 days determined within the present study would be expected to increase this.

Using the delay phase of 14d demonstrated by Kempf & Todd (1989), Todd *et al.*, (1994) predicted *Adalaria proxima* to display genetic differentiation on a scale of  $10^2$ - $10^3$  km. Additional support for the predicted larval dispersal ability was interpreted by the incidence of colour polymorphism displayed by *A. proxima* over its distributional range. The yellow mantle of individuals from western sites (Wales, N. Ireland, and the Scottish west coast) is replaced by a white form in north-west Sutherland and the east coast (Fife and Yorkshire) (Thompson & Brown, 1976; Hayward *et al.*, 1990; Todd *et al.*, 1994). In a genetic survey of 19 U.K. populations, covering a distance of 1500km from Menai Bridge, Wales to Robin Hoods Bay, Yorkshire, Todd *et al.* (1994) scored the allele frequencies of individuals at five polymorphic loci. Using hierarchical F-statistics the authors tested the observed allele frequencies for departure from Hardy-Weinburg equilibrium. The heterozygosity of individuals within sub-populations ( $F_{IS}=0.0020$ ) indicated there to be no inbreeding within populations, whilst the overall inbreeding coefficient ( $F_{IT}=0.3004$ ) was interpreted to preclude the presence of a single panmictic breeding population. The fixation index ( $F_{ST}$ ) was introduced by Wright (1923) and is considered an indication of the genetic differentiation between sub-populations (see also Wright, 1951, 1978). In a qualitative guide to the interpretation of  $F_{ST}$ , Wright (1978) proposed  $F_{ST} < 0.05$  to be indicative of 'little' genetic differentiation between populations whilst an  $F_{ST} > 0.25$  was considered to be indicative of a 'very great' degree of genetic differentiation. The  $F_{ST}$  of 0.2990 obtained by Todd *et al.* (1994) was therefore interpreted to reflect a high degree of fixation for alternative alleles between sub-populations and thus a considerable amount of genetic differentiation between populations over the geographic range considered.



*Adalaria proxima* also displays high degrees of genetic differentiation over considerably smaller distances. For example, the genetic profiles of four Scottish populations monitored over three generations show a high degree of differentiation between alleles of four loci in populations just 3km apart. Moreover, investigations on the Argyll (west Scotland) coast by Todd *et al.* (1994) indicated significant genetic differentiation between populations situated less than 100m apart.

Evidence of such small scale genetic differentiation conflicted markedly with predictions of delay capacity in *Adalaria proxima* based upon the laboratory-determined delay phase observed by Kempf & Todd (1989) and within the present study. Gene flow, and consequently, the genetic homogeneity of populations is proposed to be increased by larval dispersal (Scheltema, 1971; Berger, 1973; Crisp, 1974, 1978; Scheltema, 1978; Hedgecock, 1986). Several theories, none of which may necessarily be mutually exclusive, were advanced by Todd *et al.* (1988) in order to explain the unexpected high degree of heterogeneity, and by implication, low degree of gene flow between tidally connected populations of *A. proxima*. The authors suggested that larval transport may occur between populations, but, because of the strong local tidal currents (6 knots [Anon, 1974]) larval transport could be asymmetrical or possibly unidirectional. Alternative explanations are provided by the occurrence of post-settlement selection. For example, if sites were subject to uniform migration from one source site, differential selection processes between sites could result in the observed differentiation. Alternatively, Todd *et al.* (1988) proposed that post-settlement selection could be genotype-specific and result in selection against migrating larvae. However, both Todd *et al.* (1988) and Todd *et al.* (1994) concluded the most plausible explanation of the observed heterogeneity to be a lack of gene flow between populations caused by the omission of the pelagic phase by larvae, such that settlement would occur before dispersal.

The present study has demonstrated empirically that *A. proxima* displays a reduction in selected larval fitness components after having undergone an extended pelagic phase (Chapter 5). Established consequences of a prolonged pelagic phase included increased larval mortality, decreased metamorphic success and a reduced size on attainment of metamorphosis. This present study therefore proposes an additional hypothesis to that proposed by Todd *et al.* (1988, 1994) in order to explain the unexpectedly high degree of differentiation between geographically proximate populations. Larvae and resultant metamorphs which have undergone an extended delay phase may possess reduced fitness, and migrants may thus be at a

competitive disadvantage relative to resident larvae. Whilst the observed interpopulation variation may primarily be a reflection of a limited effective dispersal potential in *A. proxima*, it may be that reduced competitive ability of migrants is also ecologically relevant. Bearing in mind the considerable variation in the length of the precompetent period and ability to delay metamorphosis displayed by *A. proxima* larvae it is probable that these two hypotheses are not mutually exclusive. Those larvae which prolong the competent phase for a period sufficient to migrate are likely to become subject to restricted competitive ability and inferior fitness (at the settlement or post-settlement phase) and consequently immigrants would make an insignificant contribution to the genetic pool. Therefore, the actual effective dispersal range of *A. proxima* larvae is likely to be minimal in comparison to that expected from the laboratory derived maximum delay phase.

Delay phase has long been a focus of study within marine larval ecology and there exist within the literature numerous examples of dispersal potentials inferred from laboratory derived maximum delay capacities (see Scheltema, 1971; Crisp, 1974; Scheltema, 1974; Pechenik, 1980, 1984; Crisp, 1986; Pechenik, 1990). In contrast, primarily because of considerable logistical problems, the actual dispersal ability of invertebrate larvae has been directly determined for only a few species. In one of the few reported studies Olson & McPherson (1987) observed that larval behaviour shortened the delay phase of the tropical ascidian *Lissoclinum patella* from a possible 2 h to less than ten minutes. The result was a considerable disparity in potential and actual larval dispersal - from a predicted several hundred m to less than ten. The results of the present study would indicate that such behaviour may also be exercised *Adalaria proxima* larvae. Further support that the larva may settle and metamorphose within the immediate area is offered by the relatively stringent metamorphic cue requirements established within the present study. Such cue specificity would be expected to increase the risk of larval dispersal into unsuitable habitats.

The observed disparity between predicted and effective dispersal capacity apparent within this study would provide further support that caution should be exercised when considering the larval dispersal potential of species when predicted from empirically determined maximum delay capacities.

It is notable that larvae of the tropical aeolid nudibranch *Phestilla sibogae* become refractive to the natural cue, a water soluble substance emanating from the primary adult prey item *Porites compressa* (Hadfield, 1984; Hadfield & Scheuer,

1985). This inhibition of metamorphosis - termed habituation - is reversible, and caused only when larvae are exposed to the cue prior to the attainment of competence. Dehabituation requires 5-24 hr exposure to cue-free water and Hadfield & Scheuer (1985) interpreted this imposition of a minimum pelagic period to be a mechanism of ensuring dispersal from the immediate vicinity. No such habituation response to the natural cue has been documented, or observed to occur in *A. proxima*, although there has been no quantitative investigation of habituation in this species.

Several authors (Strathmann, 1974, 1985; Todd *et al.* 1988; Kempf & Todd, 1989) have proposed dispersal to be merely an unavoidable consequence of the pelagic larval phase and not, as has historically been generally accepted, the primary rôle of the pelagic larva. Evidence provided by the genetic surveys of previous authors (Havenhand, 1986; Havenhand *et al.*, 1986; Todd *et al.*, 1988, 1994) and the punitive consequences of an extended pelagic period upon fitness established within this study would suggest that the larvae of *Adalaria proxima* do not fulfil a primarily dispersive rôle.

It is notable that within the present study larval feeding did not confer any significant advantage in ability to delay metamorphosis. This may be interpreted as further support for the non-adaptive nature of the pelagic period in *A. proxima*. Facultative planktotrophy has been documented both to extend the capacity to delay metamorphosis (Perron, 1981; Kempf & Hadfield, 1985; Miller, 1993), and to reduce the negative effects of such a delay on larval and juvenile fitness among gastropod larvae (Miller, 1993). The facultatively planktotrophic larvae of the nudibranch *Phestilla sibogae* may, for example, extend the period of the delay phase up to 90% longer than starved conspecifics (Kempf & Hadfield, 1985). Like the larvae of *P. sibogae*, the pelagic larvae of *A. proxima* possess a functional digestive system similar to that of obligate planktotrophic species, and have been demonstrated to be capable of feeding (Kempf & Todd, 1989). However, in marked contrast to the larvae of *P. sibogae*, which may successfully meet all energetic requirements by feeding (Kempf & Hadfield, 1985), *A. proxima* may supplement only 34% of metabolic demands through ingestion of particulate food (Kempf & Todd, 1989). The results of the present study, that feeding neither extends, nor reduces the negative effects of, a delay in metamorphosis, indicate the functional significance of such a restricted ability to supplement endogenous energetic reserves. Whilst larvae of *P. sibogae* may be regarded as true facultative planktotrophs, larvae of *A. proxima* may more accurately be termed restricted

facultative planktotrophs. The findings of the present study offer additional support that advantage of possessing a functional digestive system in *A. proxima* is not conferred during the larval phase, but rather in overcoming the prey-size constraint encountered by the post-metamorphic juvenile (see Todd, 1986).

#### The Significance Of Interpopulation Variation In *Adalaria Proxima*

Significant interpopulation variation in the allocation of effort to fundamental reproductive parameters such as egg size and egg number were evident between the U.K. *Adalaria proxima* populations detailed within the present study. Such intraspecific variation in reproductive traits is consistent with the observed degree of genetic differentiation displayed by populations of *A. proxima* and reported of previous authors (Havenhand[1986], Todd *et al.* [1988, 1994]).

Whilst gene flow across different populations may be of value in damping the effects of acute ephemeral conditions (Strathmann, 1982) it may, in some species, be maladaptive in precluding genetic divergence in response to the local environment and consequently result in progeny possessing reduced fitness (Hedgewood, 1986). Although selection acts upon the individual, it is only at the population level that the measurement of life history strategies may be performed (Stearns, 1976). Therefore, for any relationship between egg size and another trait to be considered of ecological relevance it must be evident at the population level (Kaplan, 1980). Within the present study, considerable, and consistent, differences in spawn mass egg size, fecundity, and larval size were observed between *Adalaria proxima* originating from different sites. However, analysis of the causal mechanisms and the significance of such variation in reproductive traits between populations is problematic. As Strathmann (1977) observed 'Mother Nature...allows multiple functions for multiple interrelated traits and allows accidents of ancestry to place limits on adaptive variation'. The ecological significance of an adaptationist explanation for the observed interpopulation variation may only be evaluated by further comprehensive study of the selection regimes to which each population is subject, and manipulative breeding trials to determine the presence of outbreeding depression.

Todd *et al.* (1994) considered it to be improbable that such interpopulation heterogeneity could be a resultant of differential selection regimes between sites. The majority, if not all, of the observed heterogeneity between populations was



instead regarded as being selectively neutral, and to have been primarily influenced by population bottlenecks or by the founder effect. The neutrality hypothesis (Kimura, 1968, 1983; see also Nei, 1987) proposed variation in allozyme loci to be the resultant of the processes of mutation and random genetic drift upon selectively neutral allozymes. Variation in allele frequencies also been documented in other marine invertebrate species (Hedgecock, 1982; Buroker, 1983; Burton, 1983; Hedgecock, 1986, 1994). In a similar manner to the conclusions of Todd *et al.* (1988, 1994), a high degree of differentiation in allele frequencies between populations of the oyster *Crassostrea virginica* was attributed by Hedgecock *et al.* (1992) not to selective processes, but rather to genetic drift within subpopulations which are demarcated by larval retention. Thus it may be that the variation in reproductive traits observed between populations of *Adalaria proxima* within the present study may constitute a reflection of the process of genetic drift rather than selective forces.

The present results may also be interpreted to proffer support for additional, possibly supplementary, explanation of the apparent lack of larval migration between populations. The findings of the present study concur with those of previous authors in predicting the effective dispersal potential of *A. proxima* larvae to be more limited than would be inferred by laboratory study of delay capacity. It may be however that there is considerable variation between larvae in realized pelagic period, and whilst many settle near the point of origin, a few may remain in the water column and become subject to dispersal. The demonstrated loss of fitness by larvae and reduced size of metamorphs having been subject to the extension in pelagic phase necessary for such dispersal would intimate that the lack of genetic exchange between populations may not be credited solely to a minimal pelagic phase. Rather, migrants, having undergone the physiological effects with a delay of metamorphosis, may be at a competitive disadvantage relative to larvae of the local population which have settled soon after attainment of competence. In addition, were larvae to be subject to differential post-settlement regimes between sites, then any pre-existing competitive disadvantage possessed by immigrants may be expected to become amplified. Thus, the restricted interpopulation exchange between populations of *A. proxima* may not solely be attributable to a single exclusive mechanism (that of abbreviated larval dispersal) but may instead be the product of a generally restricted pelagic phase in combination with reduced pre- and post-settlement success of migrants due to the physiological effects of an extended delay phase.

## Conclusion

With the exception of the land snail *Arianta arbustorum* (Baur, 1994) there is a notable paucity of studies concerning intraspecific variation in life history traits across populations within the Gastropoda, and in particular a complete absence of datum regarding the Nudibranchia. Considerable variation in the allocation of reproductive resources was observed both within and between United Kingdom populations of *Adalaria proxima* surveyed within the present study. A consideration of the sources of such variation is instructive when considering the pressures affecting reproductive traits at the intraspecific level. The pattern of change in reproductive output over the course of the spawning season notably did not differ between populations. The intrapopulation decrease in spawn mass egg size, number and hatching success by adults appeared universal. That these intrapopulation trends were uniform may be indicative of the relative importance of phylogenetic constraints acting upon reproductive output at this level.

*Adalaria proxima* represents one of the most comprehensively studied nudibranchs in relation to life history variation, and indeed constitutes the only nudibranch for which genetic population profiles have been reported within the literature (Havenhand [1986], Todd *et al.* [1988, 1994]). The present study supplies empirical evidence of considerable, and consistent, differences in spawn mass egg size, fecundity, and larval size between *Adalaria proxima* originating from different sites. In addition, the quantitative analysis of the effects of a prolonged pelagic period on larval fitness, and therefore the ecological significance of delayed metamorphosis, have facilitated an estimation of the effective dispersal potential of *A. proxima*. It is this actual effective dispersal potential which is pertinent when considering the gene flow between populations (Endler, 1977). Interpretation of the causative processes and functional significance of the variation in selected reproductive parameters between populations is complex. However, evidence would suggest that over the geographic range surveyed such differentiation between populations of *A. proxima* may be most probably constitute a primarily non-adaptive phenomenon attributable to genetic drift within populations. The predicted reduced fitness and competitive ability of migrants may offer an additional mechanism to that of abbreviated larval dispersal in ensuring the maintenance of these essentially closed populations. The pre-existing body of biochemical genetic data provided by Havenhand (1986), Todd *et al.*, (1988, 1994) therefore complements the findings of the exclusively ontogenetic approach pursued within this study. Such a comprehensive body of data over a selected geographic area is

atypical among the Nudibranchia. *A. proxima* may therefore be considered an excellent candidate for further investigation of the ecological significance intraspecific variation in the allocation of reproductive resources and the biogeographical, ecological and evolutionary consequences of larval type.



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